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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 25
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

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Bogotá, D.C.,

Abogado(a)/Señor(a)
CARLOS REINALDO OLARTE GARCIA

Asunto: Radicación: 11-168961- -8
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 12622
Folios: 25

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/EP2009/004112				
Fecha de Presentación Internacional	08/06/2009				

1. Documentos estudiados

Descripción:	Radicado N°11-168961-00000-0000	Diciembre 7 de 2011
Reivindicaciones	Radicado N°11-168961-00000-0000	Diciembre 7 de 2011
	Radicado N°11-168961-00007-0000	Mayo 29 de 2014
Secuencias	Radicado N°11-168961-00000-0000	Diciembre 7 de 2011
Dibujos	Radicado N°11-168961-00000-0000	Diciembre 7 de 2011
Respuesta del solicitante	Radicado N°11-168961-00007-0000	Mayo 29 de 2014

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga

más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

Se realizará el estudio de las reivindicaciones 1 a 10 presentes en el Radicado N° 11-168961-00007-0000, porque el valor de estas reivindicaciones se encuentra incluido en la tasa de solicitud inicial.

Título de la solicitud: “Síntesis de HMO”.

El problema planteado en esta solicitud consiste en lograr un proceso alternativo para obtener oligosacáridos HMO en concentraciones mayores con respecto a las obtenidas de la leche humana.

Para resolver este problema técnico, la solicitud en estudio plantea un proceso para obtener oligosacáridos mediante una célula recombinante que contiene un ácido nucleico que codifica una proteína transportadora.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N1/00; C12N15/63.

4. De la Solicitud de la Patente

Título

El título de la solicitud se considera muy general y ambiguo porque al mencionar “síntesis de HMO” se incluye muchos métodos posibles para obtener muchos oligosacáridos posibles, pero que no están soportados en el memorial descriptivo. El objeto de la solicitud está especialmente dirigido a la obtención de fucosil lactosa donde intervienen ciertas enzimas específicas. Se solicita modificar el título de la solicitud, de tal manera que en este se menciona de manera detallada el objeto de la solicitud.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no es concisa, porque al mencionar “al menos comprende una secuencia de ácido nucleico que codifica fucosil transferasa” y “que comprende una secuencia que codifica una familia de transportadores de exportación de azúcares o un homólogo o un derivado de esta”, debido a que se incluye una cantidad amplia de posibles secuencias de nucleótido, pero que no se encuentran sustentadas en la descripción. Se solicita mencionar específicamente las secuencias nucleótidas que permiten codificar las enzimas y transportadores mencionadas en la solicitud.

Para las reivindicaciones 2 y 3 se solicita mencionar las modificaciones que se le realizan a dicha célula, así como se solicita mencionar específicamente cuales son los derivados del transportador SetA.

De igual manera, se solicita mencionar las secuencias específicas que codifiquen las enzimas mencionadas en las reivindicaciones 4 y 5.

Para el método de la reivindicación 6 se solicita mencionar la secuencia nucleótida que codifica la enzima y el transportador para el paso (a), de acuerdo a lo requerido en la reivindicación 1.

Se sugiere eliminar la reivindicación 7 y el objeto de esta reivindicación se puede adicionar al paso (b) del proceso.

Se sugiere eliminar las reivindicaciones 9 y 10, incluyendo el objeto de dichas reivindicaciones dentro el paso (a) de la reivindicación 6.

5. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación internacional: El 08 de junio de 2009.

Consultadas las fuentes de información con que se cuenta, se encontraron los siguientes documentos que anticipa el objeto de esta solicitud:

Item	Documento	Título	Fecha de Publicación
D4	US2008/0145899	"Production of oligosaccharides by microorganisms"	19/junio/2008
D5	<i>Glycoconjugate Journal</i> . 2001. 18; 465 – 474	"In vivo fucosylation of lacto-N-neotetraose and lacto-N-neohexaose by heterologous expression of Helicobacter pylori α -1,3 fucosyltransferase in engineered Escherichia coli"	30/Junio/2001
D6	<i>Journal of Biological Chemistry</i> . 1999. 274 (33); 22977 – 22984	"Functional and Biochemical Characterization of <i>Escherichia coli</i> Sugar Efflux Transporters"	13/Agosto/1999

El documento D4¹ divulga la síntesis enzimática de oligosacáridos y la obtención de precursores de los mismos mediante células recombinantes (Pág. 1, párr. [0002]).

El documento D5² divulga la síntesis de oligosacáridos en E. coli, donde la reacción de fucosilación se lleva a cabo mediante una fucosil transferasa (Pág. 465, abstract).

El documento D6³ divulga una familia de transportadores de expulsión de sacáridos (SET) los cuales expulsan compuestos sacáridos cuando hay sobresaturación celular de estos mismos o por obtención de compuestos no metabolizables (Pág. 22984, abstract y col. der., ln. 3 – 26).

6. Examen de Patentabilidad.

Nivel Inventivo (Art 18 D 486)

La reivindicación 1 divulga *"Una célula bacteriana transformada que puede ser cultivada de manera estable en un medio, modificada para producir fucosil lactosa, que comprende al menos una secuencia de ácido nucléico que codifica fucosil transferasa, donde la célula bacteriana también comprende una secuencia de ácido nucléico que codifica un transportador que participa en la exportación de azúcares (SET) o un homólogo o derivado de esta"* (Pág. 11 del Radicado N°11–

1 http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=US&NR=2008145899A1&KC=A1&FT=D&ND=3&date=20080619&DB=worldwide.espacenet.com&locale=es_LP
2 <http://www.jbc.org/content/274/33/22977.full.pdf+html?sid=8f0a738d-fcde-46f6-a84c-17fc0495fa09>
3 http://download.springer.com.ezproxy.unal.edu.co/static/pdf/720/art%253A10.1023%252FA%253A1016086118274.pdf?auth66=1411076376_f3d3759f9396ae637744b32154195af0&ext=.pdf

168961-00007-0000).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Solicitud	D4	D6
Una célula bacteriana modificada	Una célula que puede ser <i>E. coli</i> (Pág. 1, párr. [0006]; pág. 2, párr. [0019])	Una célula bacteriana que corresponde a <i>E. coli</i> (Pág. 22977, col. der., ln. 3 – 4)
Produce fucosil lactosa	Puede producir fucosil lactosa (Pág. 2, párr. [0020] – [0021]; pág. 33, párr. [0225])	No se menciona
Comprende una secuencia de ácido nucléico que codifica fucosil transferasa	Se incorpora un gen que codifica α – 1, 3 – fucosil transferasa aislado de <i>Helicobacter pylori</i> (Pág. 33, párr. [0226])	No se menciona
Comprende una secuencia de ácido nucléico que codifica un transportador de exportación de azúcares	No se menciona	Contiene cadenas de nucleótidos que codifican transportadores de sacáridos SetA, SetB, SetC (Pág. 22978, tabla I)

El documento D4 se considera el estado de la técnica más cercano a la célula modificada, de acuerdo a lo descrito en la reivindicación 1, porque se menciona una célula modificada (preferiblemente *E. coli*) a la cual se le inserta una secuencia de nucleótido que codifica fucosil transferasa para obtener sacárido derivado de fucosa que se puede adherir a un disacárido de lactosa (Pág. 1, párr. [0006]; pág. 2, párr. [0019] – [0021]; pág. 33, párr. [0225] – [0226]).

La diferencia entre la célula descrita en la solicitud con respecto a la célula divulgada en el documento D4, radica en que la célula modificada de la solicitud contiene adicionalmente una secuencia de ácido nucléico que codifique una proteína transportadora que participa en la exportación de los sacáridos.

Esta diferencia le confiere el efecto técnico de obtener el compuesto sacárido (fucosil lactosa) sin la necesidad de inducir la lisis celular, de tal manera que el sacárido obtenido no se contamine por material proveniente de la célula de la célula.

El problema técnico objetivo que se pretende resolver es el siguiente: Cómo modificar la célula divulgada en el documento D4, para obtener una célula modificada alternativa que sea capaz sintetizar compuestos sacáridos.

El documento D6 divulga una célula modificada de *E. coli* que comprende la inclusión de secuencias de ácido nucléico que codifican las proteínas transportadoras de azúcares SetA, SetB y SetC con el propósito de eliminar el exceso de sacáridos (en este caso glucosa) que no permiten aprovechar las glucosa – 6 – fosfato para el metabolismo normal celular (Pág. 22977, col. der.; pág. 22978, tabla I).

Por lo tanto, una persona normalmente versada en la materia y conocedora de los conceptos mencionados en los documentos D4 y D6, sin ningún esfuerzo, modificaría la célula descrita en el documento D4, incluyendo la inserción de las secuencias de ácido nucléico que codifican las proteínas transportadoras SetA, SetB y SetC para eliminar el exceso de sacárido obtenido, tal como lo sugiere el documento D6, para obtener la célula modificada propuesta en esta solicitud, de manera obvia o evidente.

En consecuencia, la reivindicación 1, de acuerdo con los documento D4 y D6, carece de Nivel Inventivo.

Las reivindicaciones 2 a 10, dependientes de la reivindicación 1, no divulgan características técnicas adicionales que confieran nivel inventivo al proceso de la reivindicación 1, porque el documento D4 sugiere un método para la producción de fucosiltransferasa, que consiste en cultivar las células modificadas en un medio que contiene sustrato (que puede ser fucosa) en un reactor y extraer el sacárido obtenido (Pág. 1, párr. [0014]; pág. 3, párr. [0035]; pág. 33, párr. [0224] – [0227]; pág. 39, párr. [0274]). Por lo tanto, estas reivindicaciones, también carecen de Nivel Inventivo.

La reivindicación 1 divulga “Una célula bacteriana transformada que puede ser cultivada de manera estable en un medio, modificada para producir fucosil lactosa, que comprende al menos una secuencia de ácido nucleíco que codifica fucosil transferasa, donde la célula bacteriana también comprende una secuencia de ácido nucleíco que codifica un transportador que participa en la exportación de azúcares (SET) o un homólogo o derivado de esta” (Pág. 11 del Radicado N°11–168961–00007–0000).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Solicitud	D5	D6
Una célula bacteriana modificada	Una célula modificad que corresponde a <i>Helicobacter pylori</i> (Pág. 466, col. izq., ln. 35 – 39)	Una célula bacteriana que corresponde a <i>E. coli</i> (Pág. 22977, col. der., ln. 3 – 4)
Produce fucosil lactosa	Producción de oligosacáridos fucosilados que contiene lactosa como los compuestos 1, 2 y 3 (Pág. 467, col. der., ln. 23 – 35; col. der., ln. 39 – 53)	No se menciona
Comprende una secuencia de ácido nucleíco que codifica fucosil transferasa	Comprende una secuencia que codifica fucosil transferasa, obtenida de <i>E. coli</i> (Pág. 466, col. izq., ln. 35 – 39)	No se menciona
Comprende una secuencia de ácido nucleíco que codifica un transportador de exportación de azúcares	No se menciona	Contiene cadenas de nucleótidos que codifican los transportadores SetA, SetB, SetC (Pág. 22978, tabla I)

El documento D5 se considera el estado de la técnica más cercano a la célula modificada, de acuerdo a lo descrito en la reivindicación 1, porque se menciona una célula modificada de *Helicobacter pylori* a la cual se le inserta una secuencia de nucleótido extraída de *E. coli*, que codifica fucosil transferasa, para obtener sacáridos de fucosil lactosa (Pág. 466, col. izq., ln. 35 – 39; pág. 467, col. der., ln. 23 – 35; col. der., ln. 39 – 53).

La diferencia entre la célula descrita en la solicitud con respecto a la célula divulgada en el documento D5, radica en que la célula modificada de la solicitud contiene adicionalmente una secuencia de ácido nucleíco que codifique una proteína transportadora que participa en la exportación de los sacáridos.

Esta diferencia le confiere el efecto técnico de obtener el compuesto sacárido (fucosil lactosa) sin la necesidad de inducir la lisis celular, de tal manera que el sacárido obtenido no se contamine por material proveniente de la célula de la célula.

El problema técnico objetivo que se pretende resolver es el siguiente: Cómo modificar la célula divulgada en el documento D5, para obtener una célula modificada alternativa que sea capaz sintetizar compuestos sacáridos.

El documento D6 divulga una célula modificada de E. coli que comprende la inclusión de secuencias de ácido nucléico que codifican las proteínas transportadoras de azúcares SetA, SetB y SetC con el propósito de eliminar el exceso de sacáridos (en este caso glucosa) que no permiten aprovechar las glucosa – 6 – fosfato para el metabolismo normal celular (Pág. 22977, col. der.; pág. 22978, tabla I).

Por lo tanto, una persona normalmente versada en la materia y conocedora de los conceptos mencionados en los documentos D5 y D6, sin ningún esfuerzo, modificaría la célula descrita en el documento D5, incluyendo la inserción de las secuencias de ácido nucléico que codifican las proteínas transportadoras SetA, SetB y SetC para eliminar el exceso de sacárido obtenido, tal como lo sugiere el documento D6, para obtener la célula modificada propuesta en esta solicitud, de manera obvia o evidente.

En consecuencia, la reivindicación 1, de acuerdo con los documento D5 y D6, carece de Nivel Inventivo.

Las reivindicaciones 2 a 10, dependientes de la reivindicación 1, no divulgan características técnicas adicionales que confieran nivel inventivo al proceso de la reivindicación 1, porque el documento D5 sugiere un método de obtención del sacárido mediante el cultivo de las células modificadas en un reactor, seguido de la extracción del sacárido obtenido (Pág. 471 y 472). Por lo tanto, estas reivindicaciones, también carecen de Nivel Inventivo.

7. Conclusión

Los requisitos de Patentabilidad de esta solicitud se ven afectados de la siguiente manera:

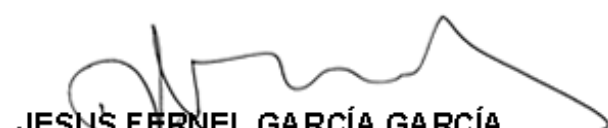
Item	Documento	Reivindicaciones	Requisitos que afecta
D4	US2008/0145899	1 a 10	Nivel Inventivo
D5	<i>Glycoconjugate Journal</i> . 2001. 18; 465 – 474	1 a 10	Nivel Inventivo
D6	<i>Journal of Biological Chemistry</i> . 1999. 274 (33); 22977 – 22984	1 a 10	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JESÚS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-10-07

Elaboró: Dayron Alexander Ramirez Reyes
Revisó: Sandra Carolina Crespo



In vivo fucosylation of lacto-*N*-neotetraose and lacto-*N*-neohexaose by heterologous expression of *Helicobacter pylori* α -1,3 fucosyltransferase in engineered *Escherichia coli*

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We report here the *in vivo* production of type 2 fucosylated-*N*-acetyllactosamine oligosaccharides in *Escherichia coli*. Lacto-*N*-neofucopentaose Gal β 1-4GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)Glc, lacto-*N*-neodifucohexaose Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)Glc, and lacto-*N*-neodifucooctaose Gal β 1-4GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)Glc were produced from lactose added in the culture medium. Two of them carry the Lewis X human antigen. High cell density cultivation allowed obtaining several grams of fucosylated oligosaccharides per liter of culture. The fucosylation reaction was catalyzed by an α -1,3 fucosyltransferase of *Helicobacter pylori* overexpressed in *E. coli* with the genes *lgtAB* of *N. meningitidis*. The strain was genetically engineered in order to provide GDP-fucose to the system, by genomic inactivation of gene *wcaJ* involved in colanic acid synthesis and overexpression of *RcsA*, positive regulator of the colanic acid operon.

To prevent fucosylation at the glucosyl residue, lactulose Gal β 1-4Fru was assayed in replacement of lactose. Lactulose-derived oligosaccharides carrying fucose were synthesized and characterized. Fucosylation of the fructosyl residue was observed, indicating a poor acceptor specificity of the fucosyltransferase of *H. pylori*.

Keywords: *Escherichia coli*, fucosylation, Lewis X, metabolic engineering, oligosaccharides

Abbreviations: CO5, chitopentaose; Fuc, fucose; Fru, fructose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; HMO, Human Milk Oligosaccharide; Le^X, Lewis X; LNnT, Lacto-*N*-neotetraose; LNnFP, lacto-*N*-neo-fucopentaose; LNnDFH, lacto-*N*-neo-di fuco-hexaose; LNnT^U, lacto-*N*-neo-tetraose analog in which fructose replaces glucose; LNnH^U, lacto-*N*-neo-hexaose analog in which fructose replaces glucose.

Introduction

L-fucose is commonly found in complex carbohydrates and plays a major role in many processes of biological recognition. The availability of large amounts of fucosylated oligosaccharides would make them useful as precursors or ready-to-use drugs for fundamental investigative research as well as for therapeutic trials. The chemical synthesis of fucose-containing oligosaccharides such as ABH and lewis antigens has long been achieved [1], but this technology is expensive and time-consuming. Enzymatic synthesis offers a seductive alternative

way to the synthesis of oligosaccharides; and indeed the *in vitro* enzymic synthesis of Le^X has been reported [2]. Nevertheless, the cost of GDP- β -L-fucose, which is the activated sugar nucleoside-sugar precursor used by fucosyltransferase, is a limiting factor for large scale application.

In both prokaryotes and eukaryotes, GDP-L-fucose is biosynthesized *de novo* from mannose-6-phosphate and GTP by the successive action of four enzymes [3,4]. In *E. coli* these four enzymes are encoded by the *manB*, *manC*, *gmd* and *wcaG* genes which are part of the colanic acid gene cluster. Several processes have been designed to produce GDP-L-fucose by recombinant *E. coli* enzymes. The *in vitro* conversion of GDP-mannose to GDP-L-fucose requires an NADPH supplementation and can be achieved using purified recombinant Gmd and WcaG [5]. Taking advantage of the high cytoplasmic pool of

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Data presented at the last XVI International Symposium on Glycoconjugates (The Hague, August 2001).

GDP-mannose in *Saccharomyces cerevisiae*, Mattila and collaborators have obtained a significant production of GDP-L-fucose by a recombinant yeast strain expressing the *E. coli* *gmd* and *wcaG* genes [6].

A large scale system to produce GDP-L-fucose from GMP and mannose was also established by coupling permeabilized resting cells of *Corynebacterium ammoniagenes* and recombinant *E. coli* expressing GDP-L-fucose biosynthetic genes: GTP was produced from GMP by *C. ammoniagenes* cells and used by *E. coli* cells to convert mannose into GDP-L-fucose [7].

One of the limitation of these systems is the strong inhibition of the Gmd enzyme by the end product GDP-L-fucose [8]. This problem can be overcome by carrying out separately the last step catalyzed by the GDP-L-fucose synthase WcaG. One alternative is to couple the GDP-L-fucose synthesis with oligosaccharide fucosylation. In this respect, the living factory approach is particularly attractive. This approach consists in producing oligosaccharides in a single growing living bacteria that overexpresses recombinant glycosyltransferase genes and provides the energetic requirement for maintaining the sugar-nucleotide pool level [9].

The living factory approach has been initially developed with the chitooligosaccharide synthase NodC which has the unique property of producing chitooligosaccharides without requiring an exogenous acceptor [10]. The method has been recently extended to the synthesis of lactose-derived oligosaccharides by using β -galactoside permease to internalized lactose [11]. By this way more than 5 g l^{-1} of a mixture of lacto-*N*-neotetraose (LNnT) and lacto-*N*-neohexaose (LNnH) were obtained by cultivating at high cell density an *E. coli* strain overexpressing the *Neisseria meningitidis* genes encoding the β 1,3 *N*-acetyl glucosaminyltransferase (LgtA) and the β 1,4 galactosyltransferase (LgtB).

The α -1,3 fucosyltransferase encoded by the *fucT* gene of *Helicobacter pylori* has been successfully expressed in *E. coli* [12]. This enzyme uses *N*-acetyllactosamine and LNnT as acceptors but not lactose. An *E. coli* strain that coexpresses *fucT* and the *lgtAB* of *N. meningitidis* should thus be able to produce fucosylated derivatives of LNnT from lactose (Figure 1). We show in this paper that this production requires a metabolic engineering of the GDP-L-fucose biosynthesis pathway to increase the availability of GDP-L-fucose, which is not sufficiently produced in our fermentation conditions by regular *E. coli* strains. In addition we report the structural characterization of the different fucosylated oligosaccharides that were produced from lactose and its β -galactoside analog lactulose (4-O- β -D-galactopyranosyl-D-fructofuranose).

Results

In vivo fucosylation of lacto-*N*-neotetraose

The first attempts to detect a production of fucosylated oligosaccharides in lactose supplemented cultures of *E. coli*

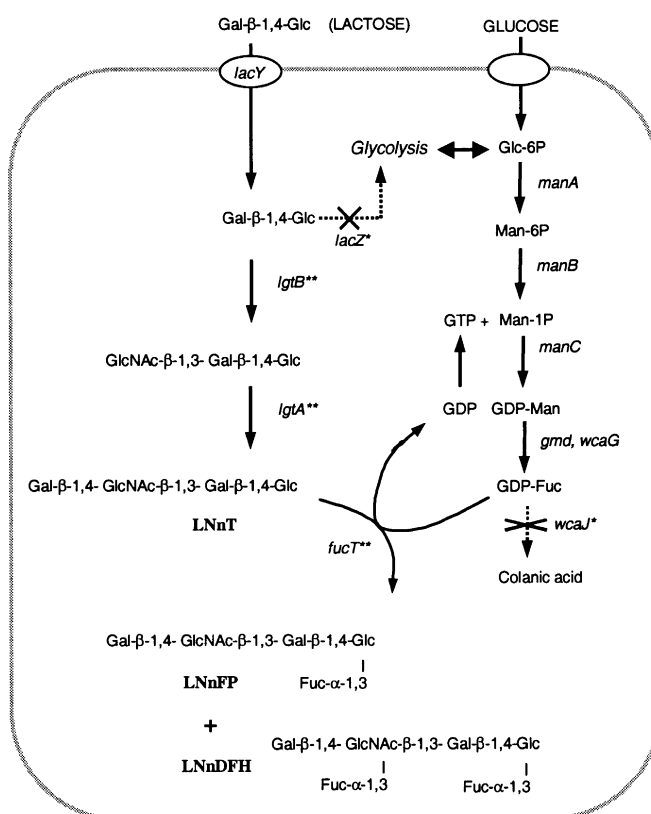


Figure 1. *In vivo* biosynthesis pathway of fucosylated lacto-*N*-neotetraose in engineered *Escherichia coli* cells. Genes encoding the enzymes involved in the different reactions are shown in italic characters. Glycosyl residues which have been underlined are potential fucosylation sites of the fucosyltransferase according to its *in vitro* activity. Lactose is internalized by specific permease LacY and cannot be degraded due to *lacZ* inactivation. It is glycosylated by GlcNAc-T and Gal-T encoded by genes *lgtAB* of *N. meningitidis*. Main final product LNnT is a possible acceptor for fucosyltransferase encoded by gene *fucT* of *H. pylori*. *Inactivated endogenous genes; **heterologous genes carried on expression vectors.

strain JM107 overexpressing the *lgtAB* and *fucT* genes were unsuccessful. *In vitro* assays of fucosyltransferase activity indicated that *fucT* was correctly expressed in *E. coli*, though the activity was considerably higher when *fucT* was under the control of the PT7 promoter ($5100 \text{ pmol.mg}^{-1}.\text{min}^{-1}$) than of the Plac promoter ($100 \text{ pmol.mg}^{-1}.\text{min}^{-1}$).

As the lack of fucosylation could be due to an insufficient level of GDP-L-fucose in the cells, we tried to increase the GDP-L-fucose availability by metabolic engineering. We used a two level strategy focused on the regulation of colanic acid biosynthesis: (i) the gene *rcaA* which encodes a positive regulator of the colanic acid operon [13] was cloned by PCR and overexpressed in a plasmid under the control of the Plac promoter. By this way the induction of *rcaA* expression by IPTG indirectly induced the expression of the GDP-L-fucose biosynthesis genes located in the colanic acid cluster- (ii) the production of colanic acid was suppressed by inactivating the

wcaJ gene, located downstream of the GDP-L-fucose biosynthesis genes in the colanic acid cluster and believed to encode the initiating glucose-1-phosphate transferase that attaches the first glucose residue on the lipid carrier intermediate [3]. This suppression allows the GDP-L-fucose biosynthesis flux to be diverted completely from colanic acid production to the synthesis of fucosylated oligosaccharide.

The efficiency of fucosylation was compared with different strains and plasmid combinations, data are summarized in Table 1. Highest fucose production was obtained with the strain col1K,DE3 (pLNTR1T, pSM9710) called Le^x cells. The absence of fucosylation in the control strain col1 (pLNTR1T alone) indicated that the fucosyltransferase encoded by *fucT* is essential for fucosylation. In addition, the very low fucosylation in the control strain col1 (pLNT1T, pSM9711), which do not overexpress *rcaA*, confirmed that an induction of the GDP-L-fucose biosynthesis genes is required to achieve an efficient fucosylation.

No significant fucosylation was observed with the strain col1 (pLNTR1T, pFUTA1) which has the *fucT* gene under the control of the Plac promoter. This result correlates the *in vitro* enzymatic assays.

Purification and analysis of fucosylated oligosaccharides produced from lactose

Seventy percent of fucose and GlcNAc containing oligosaccharides produced by Le^x cells (see Table 1) was recovered in the intracellular fraction, which was first purified by charcoal adsorption. The separation of the intracellular oligosaccharide content was then performed by gel permeation on Biogel P2. Three main oligosaccharides (compounds 1, 2, and 3) were detected and purified, besides unreacted LNnT, LNnH and lactose (Figure 2).

Compound 1—FAB-MS analysis showed a molecular ion $[M+H]^+$ of 854 compatible with composition Fuc₁Gal₂GlcNAcGlc. The ¹H-NMR spectrum by comparison with the

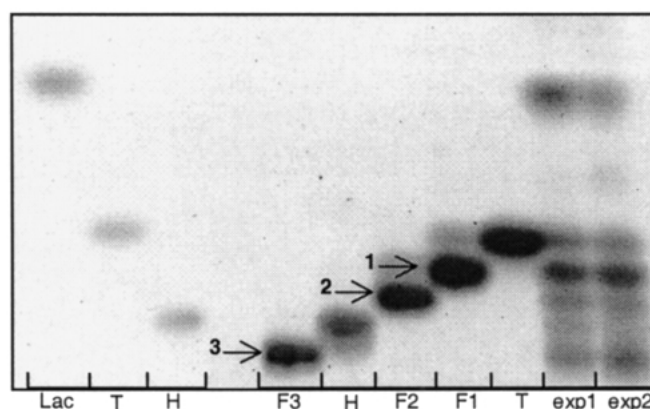
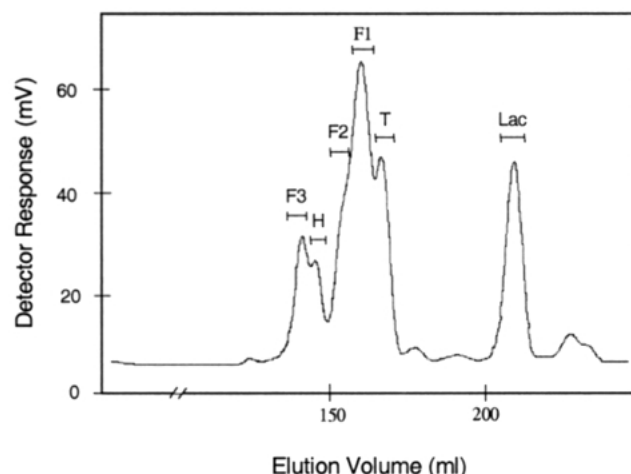


Figure 2. Separation of oligosaccharides produced by Le^x cells. Upper panel: Bio-Gel P-2 chromatography. Extracted oligosaccharides from activated charcoal where analysed on a calibrated Bio-Gel P-2 column and monitored with an in-line differential refractometer. Lower panel: TLC plate analysis of Bio-Gel P-2 fractions. Legend: T, Lacto-*N*-neotetraose; H, Lacto-*N*-neohexaose; exp1-2, intracellular cells contents from two independent experiments. Compounds 1, 2, and 3 (black arrows) were recovered from corresponding F1, F2 and F3 fractions and subsequently purified as described in Materials and Methods.

Table 1. Rate of fucosylation in *coli* strains with different expression vectors

Strain	Plasmids	Genes	Intracellular fucosylation (Fuc g/l)
col1K	pLNTR1T	<i>lgtA</i> , <i>lgtB</i> , <i>rcaA</i>	—
col1K	pLNTR1T	<i>lgtA</i> , <i>lgtB</i> , <i>rcaA</i>	0.01
	pFUTA1	<i>fucT</i> (<i>lac</i> promoter)	
col1K, DE3*	pLNT1T	<i>lgtA</i> , <i>lgtB</i> ,	0.05
	pSM9711	<i>fucT</i> (T7 promoter)	
col1K, DE3*	pLNTR1T	<i>lgtA</i> , <i>lgtB</i> , <i>rcaA</i>	0.70
	pSM9710	<i>fucT</i> (T7 promoter)	

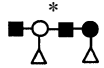
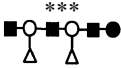
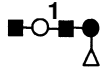
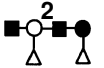
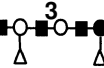
*Genomic insertion of T7 polymerase cassette.

literature (Table 2) was consistent with the proposed structure Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc.

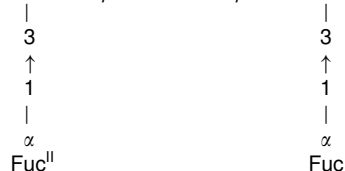
Compound 2—FAB-MS analysis showed a molecular ion $[M+H]^+$ of 1000 compatible with composition Fuc₂Gal₂GlcNAcGlc. The ¹H-NMR spectrum by comparison with the literature was consistent with the proposed structure Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc.

Compound 3—FAB-MS analysis showed a molecular ion $[M+H]^+$ of 1365 compatible with composition Fuc₂Gal₃GlcNAc₂Glc, suggesting a LNnH skeleton substituted with two Fuc residues. The ¹H-NMR spectrum confirmed the substitution of reducing Glc with one Fuc residue, but did not allow the location of the second Fuc residue to be determined. On the other hand, the FAB-MS fragmentation pattern of permethylated compound 3 indicated that the second Fuc

Table 2. ^1H -NMR analysis of oligosaccharides 1-3 produced from lactose. The legend of symbols is Δ , fucose; $\bullet$, glucose; $\blacksquare$, galactose; $\circ$, *N*-acetylglucosamine. Referenced compound data are from *[23]; **[24]; ***[14]

		Oligosaccharides						
Residues	Reporter groups							
		ppm						
α -L-Fuc (Glc)	H-1 α	5.389	5.371	5.372		5.36	5.36	5.37
	H-1 β	5.446	5.427	5.428		5.42	5.41	5.41
	H-6 α	1.193	1.167	1.163		α,β 1.16	—	α,β 1.16
	H-6 β	1.187	1.161	1.157				
α -L-Fuc ^{II} (GlcNAc ^{III})	H-1			5.135	5.111		5.12	
	H-6			1.174	1.147		—	
α -L-Fuc ^{II} (GlcNAc ^V)	H-1				5.132			5.13
	H-6				1.175			1.17
β -D-Gal ^{II}	H-1	4.437	4.415	4.414	4.432	4.41	4.4	4.41
	H-4	3.898	4.096	4.466	4.154	4.09	4.08	4.1
β -D-Gal ^{IV}	H-1		4.48	4.096	4.442	4.47	4.43	4.45
	H-4		—	3.782	4.097	3.96		4.15
β -D-Gal ^{VI}	H-1				4.464			4.48
	H-4				3.897			3.96
D-Glc	H-1 α	5.187	5.18	5.180	5.218	5.17	5.17	5.18
	H-1 β	4.658	4.65	4.651	4.66	4.64	4.64	4.65
β -D-GlcNAc ^{III}	H-1		—	4.7	4.714	4.69	4.69	4.7
	NAc-CH ₃		2.028	2.018	2.020	2.02	2.02	2.03
β -D-GlcNAc ^V	H-1				4.701			4.7
	NAc-CH ₃				2.016			2.018

Numeration of residues is: Gal^{VI}- β -1 $\rightarrow$ 4-GlcNAc^V- β -1 $\rightarrow$ 3-Gal^{IV}- β -1 $\rightarrow$ 4-GlcNAc^{III}- β -1 $\rightarrow$ 3-Gal^{II}- β -1 $\rightarrow$ 4-Glc



was attached to the proximal GlcNAc (III) (Figure 3). The presence of *m/z* fragments 464 and 668 is typical of terminal non-reducing LacNAc residue, indicating substitution on the internal GlcNAc. The proposed structure of compound **3** is therefore Gal β 1-4GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)Glc.

Assays with lactulose as acceptor

Due to the preferential fucosylation on Glc of LNnT, lactulose (Gal- β -1,4-Fru) was assayed instead of lactose in order to favor the fucosylation of LN groups i.e. the synthesis of Le^X. Without Fuc-T overexpression, incorporation of lactulose into oligosaccharides occurred at a similar rate to that of lactose (data not shown). In fucosylating strains, two major fucosylated oligosaccharides (compounds **4** and **5**) were produced.

Compound **4**—FAB-MS analysis showed a molecular ion $[\text{M}+\text{H}]^+$ of 854 compatible with composition FucGal₂Glc-

NAcFru, suggesting a LNnT^U skeleton substituted with one Fuc residue. The linkage position of the sugar residues was determined by methylation analysis (Table 3). Surprisingly, the GlcN residue is 4-substituted, and therefore cannot be fucosylated. On the other hand, the Fru derivative is 3-substituted, implying that Fuc is linked to Fru (Figure 4). The FAB-MS spectrum of permethylated oligosacchariditol (Figure 5) showed fragments at *m/z* 464, 668, and 859, consistent with a lack of fucosylation on GlcNAc. The proposed structure for compound **4** is Gal β 1-4GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)Fru.

Compound **5**—FAB-MS analysis showed a molecular ion $[\text{M}+\text{H}]^+$ of 1000 compatible with composition Fuc₂Gal₂GlcNAcFru, suggesting a LNnT^U skeleton substituted with two Fuc residues. The linkage position of the sugar residues was determined by methylation analysis. The GlcN residue is 3,4-substituted, in agreement with a Le^X motif. The Fru derivative is 3-substituted as for compound **4**, and the following structure

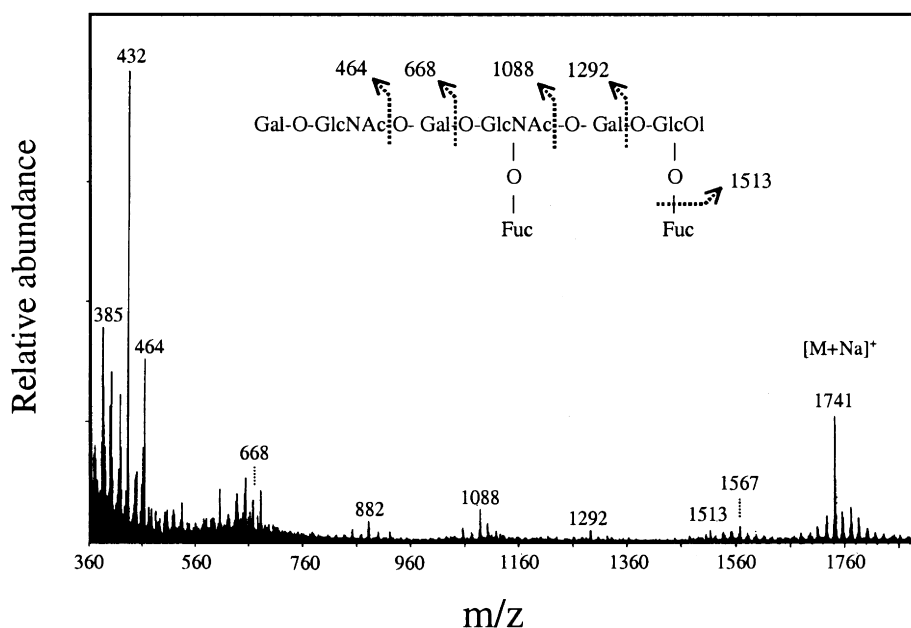


Figure 3. FAB-MS analysis of permethylated and reduced compound **3**.

Table 3. Methylation analysis of the fucosylated oligosaccharides **4** and **5** produced from lactulose

Residue	Linkage	Relative amount*	
		4	5
Fru	3-linked	1	1
Gal	terminal	1.3	1.4
	3-linked	1.2	1.3
GlcN**	4-linked	+++	+++
	3,4-linked	—	+++
Fuc	terminal	0.8	1.7

*Calculated on the base of the total ion response in GC-MS analysis and relative to the Fru residue.

**Detected on a separate column and not quantified.

for compound **5** can be proposed: Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)Fru.

Compounds **4** and **5** are structural analogs of compounds **1** and **2** produced from lactose.

Discussion

We report here the *in vivo* fucosylation of oligosaccharides in *Escherichia coli*. The substrates LNnT and LNnH were produced endogenously by overexpression of genes *lgtAB* as previously described [11]. The key point of our strategy has been to engineer an *E. coli* strain capable of furnishing GDP-fucose to the system, despite the fact it is not constitutively

produced in *E. coli*, unlike the UDP-galactose and UDP-*N*-acetylglucosamine precursors of LNnT and LNnH.

The major oligosaccharide (compound **1**) obtained from lactose is a lacto-*N*-neo-fucopentaose. It represents about 80% of the total fucosylated fraction, and its production can be approximated to 3 g/L of culture. This oligosaccharide does not carry the Le^X determinant. This result was in agreement with the known specificity of the FucT of *H. pylori*, which fucosylates the reducing glucosyl residue of LNnT more efficiently than GlcNAc [12].

Two oligosaccharides (compounds **2** and **3**) obtained from lactose do carry Le^X motifs. Compound **2** is a lacto-*N*-neodifucohexaose. It likely originates from subsequent fucosylation of compound **1** by Fuc-T. Its presence was reported in human milk, it thus belongs to the “HMO” family [14].

We attempted to improve the yield of Le^X motif production by using another β -galactoside precursor lacking glucose reducing end. Lactulose (Gal β 1,4Fru) was chosen for its low cost and its resemblance to lactose. As expected, action of LgtA and LgtB led to the endogenous synthesis of LNnT and LNnH analogs (*i.e.* LNnT^U and LNnH^U). Our wish was that only GlcNAc residue could be fucosylated, giving Le^X products. Quite unexpectedly, a fucosylation of the OH(C₃) of fructose was observed. This suggests that the position of this residue among the tetraose chain is at least as important as the nature of the residue itself in the enzyme recognition. Indeed, it suggests that the distal LN motif (in LN-Lac or LN-Lac^U) probably plays an important role in Fuc-T recognition. Fucosylation of the internal LN motif of LNnH in compound **3** argues for this hypothesis.

We describe here for the first time heterologous expression of an α -fucosyltransferase coupled to its *in vivo* catalysis in *E.*

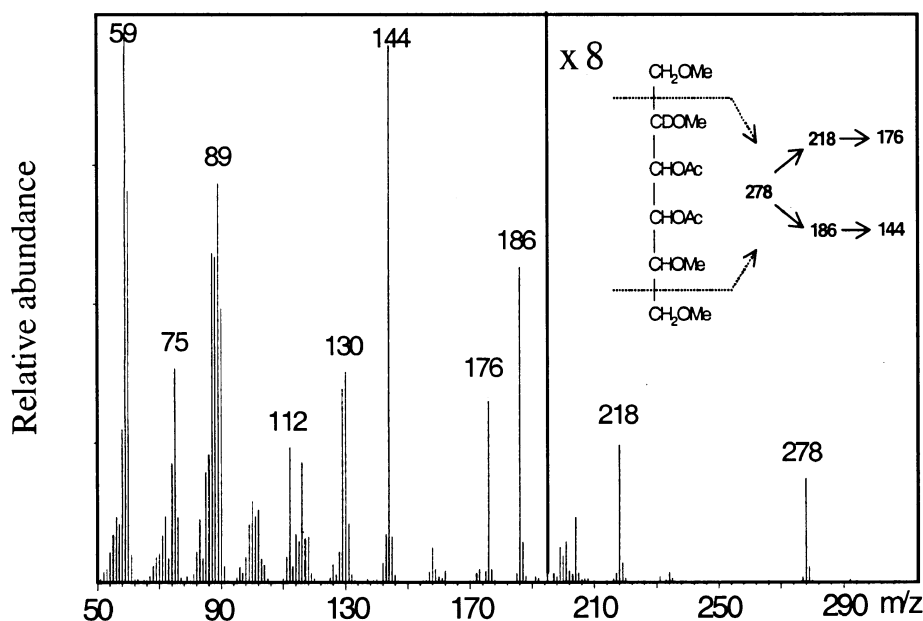


Figure 4. Electron impact fragmentation profile of partially methylated alditol acetate derivative of fructose from compounds 4 and 5. Compounds 4 and 5 were reduced with NaBD₄ before methylation. Acetylation on carbon 3 indicates substitution (i.e. fucosylation) prior to hydrolysis and acetylation.

coli cells. The *N*-acetylglucosaminyl skeleton that was used as acceptor could be modified by many other fucosyltransferases. For instance, the biosynthesis of the human H determinant Fuc- α -1,2-LacNAc- could be attempted by the same approach. The limitation for this and other determinants would be the availability of enzymes with the desired specificity which can be expressed in *E. coli*. At present, the only available bacterial α -1,2-fucosyltransferase is that of *H. pylori*, but this enzyme is almost inactive on type 2 *N*-acetylglucosaminyl acceptors *in vitro* [15].

The Le^X motifs that were synthesized are potential precursors for the synthesis of sialyl Le^X. Heterologous expression of sialyltransferase such as Nst01 of *Neisseria meningitidis*, which has been used to produce sialyllactose in *E. coli* [11], should allow the production of SLe^X motifs by a similar approach in a near future.

Materials and methods

Construction of expression plasmids

pLNT1C and pLNT1T were both plasmids providing the capability of synthesizing the type 2 poly-*N*-acetylglucosamine skeleton. They contain two genes of *N. meningitidis* MC58, namely *lgtA* encoding an β -1,3 *N*-acetylglucosaminyltransferase (genbank # 25839) and *lgtB* encoding a β -1,4 galactosyltransferase (genbank # 25839). The plasmids were constructed as follows: the gene *lgtA* together with its upstream promoter tac-tac was amplified by PCR from the expression vector pCWlgtA [16] using a pair of primers containing *Hind*III sites,

i.e. 5'-CTTTAAGCTTCCGGCTCGTATAA (sense) and 5'-GACAGCTTATCATCGATAAGCTT (antisense). After intermediate subcloning in a pPCR 2.1-TOPO vector (Invitrogen PCR kit), the *Hind*III fragment was ligated into the *Hind*III site of pBB1gtB, expression vector carrying the gene *lgtB* expressed under control of a lac promoter [11] to generate the expression vector pLNT1C, a low copy vector harboring a broad host range *ori* compatible with Col1 *ori* expression vectors, carrying *lgtA* and *lgtB* both inducible by IPTG but differentially controlled by tac-tac and lac promoters. A related expression vector pLNT1T, differing only in its antibiotic resistance gene (tetracycline instead of chloramphenicol) was obtained by subcloning the *Xho*I-*Xba*I *lgtAB* expression cassette fragment released from pLNT1C into *Xho*I-*Xba*I polylinker sites of pBBR-MCS3 [17].

pLNTR1T was made by subcloning *E. coli* gene *rcsa* in pLNT1T as follows: *rcsa* was obtained by PCR amplification from genomic DNA of strain JM109 with primers 5'-AGGGTACCCATGTTGTTCCGTTTAG (*Kpn*I, sense) and 5'-AATCTAGAGTAATCTTATTCAGCCTG (*Xba*I, antisense). After intermediate subcloning in a pPCR 2.1-TOPO vector (Invitrogen PCR kit), the 0.8 kb *Kpn*I-*Xba*I fragment was inserted between the *Kpn*I-*Xba*I sites of pBBR-MCS1 to generate pRC1C. pRC1C was linearised with *Kpn*I, blunt-ended and digested with *Xba*I in order to be inserted between the *Sma*I-*Xba*I sites of pLNT1T downstream *lgtA*. The resulting construction was named pLNTR1T.

pSM9711 contained the α 1,3-fucosyltransferase gene *fucT* (HP0379) of *Helicobacter pylori*. The *fucT* gene was amplified from *H. pylori* 26695 genomic DNA using the PCR primers

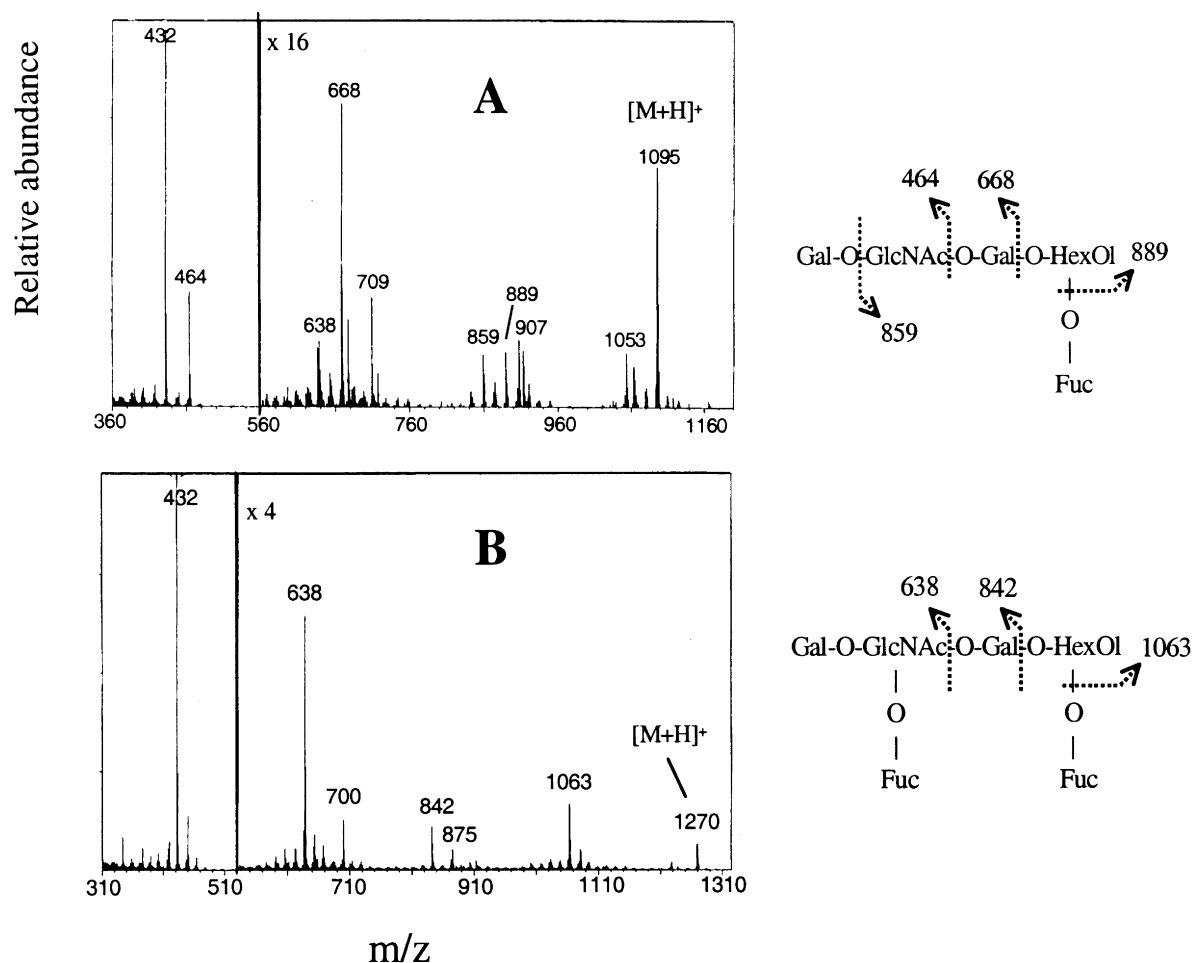


Figure 5. FAB-MS analysis of permethylated and reduced compounds **4** and **5**.

5'-TGTGCGGAGTTTGGATCCCATATGTTCCAACCCCTA-TTA (*Nde*I, sense) and 5'-GCGATGATGCGCAAGGGGTTT-GA (antisense). After intermediate cloning into pGEM-T Easy (Promega), a *Nde*I-*Eco*RI fragment containing *fucT* was excised and inserted into pET-21a (Novagen) between the *Nde*I and *Eco*RI sites, to generate pSM9710. Another construction pFUCT1 was done with a vector carrying a promoter *lac* by insertion of the *Xba*I-*Sac*I fragment of pSM9711 containing *fucT* in pUC19.

Bacterial strains

E. coli JM107 and JM109 were obtained from the Deutsche Sammlung von Mikroorganismen. Due to the $\Delta(lacZ)$ M15 deletion in the *lacZ* gene, these strains are unable to produce a complete active β -galactosidase.

Strain deficient in colanic acid synthesis (col1K) was obtained by knock-out of *wcaJ* encoding for a protein involved in the last steps of colanic acid synthesis [3]. This was carried

out by insertional inactivation of *wcaJ* with a kanamycin resistance cassette (pUC4K, Pharmacia). A DNA sequence containing *wcaJ* and some flanking DNA was amplified by PCR from *E. coli* DNA of JM109 using primers 5'-AAGTCATATCAATATGCCGCT and 5'-CCACGATC-CAGTCTCTCC. The resulting 1.8 kb fragment was cloned pTOPO2.1 (Invitrogen), released with *Eco*RI and subcloned into the *Eco*RI site of pUC19. *Eco*RI sites were subsequently methylated by treatment with *Eco*RI methylase. The vector was linearised at the *Apo*I site within *wcaJ*, into which was inserted a kanamycin resistance cassette excised from pUC4K (Pharmacia). Finally, the *Xba*I-*Xmn*I *wcaJ::KanR* fragment was moved into *Xba*I-*Sma*I polylinker sites of the suicide vector pCVD442, and subsequently used to obtain mutants from a JM107 *E. coli* strain (*recA*+) by homologous recombination as previously described [18]. Recombining mutants col1K were recovered on selective plates containing kanamycin.

Strain col1K, DE3 was prepared by genomic insertion of a DE3 cassette (T7 pol gene under control of *lacUV5* promoter) using the DE3 lysogen kit from Novagen.

E. coli strain "Le^X" was made by transformation of strain col1K,DE3 with plasmids pLNTR1T and pSM9711.

Plasmids and strains are summarized in Table 4.

High cell density cultivation

High-density cell culture was carried out in 2 liter reactors containing one liter of mineral culture medium as previously described [19]. Except where otherwise indicated the culture temperature was 28°C and the pH was 6.8. The cultivation strategy included 3 phases: a first exponential growth phase which started at the inoculation of the fermentor and lasted until exhaustion of the carbon substrate (glucose) initially added to the medium at a concentration of 17 g l⁻¹. Then, the acceptor lactose (4 g) was added together with the inducer (IPTG 50 mg l⁻¹). Medium growth rate was maintained during 5 hours by glucose supplementation at feeding rate of 3.6 g l⁻¹ h⁻¹. Finally, glucose supplementation was decreased to 1.8 g l⁻¹ h⁻¹ and a lactose supplementation of 0.45 g l⁻¹ h⁻¹ was also provided until the end of the fermentation (10–15 hours).

Table 4. *E. coli* strains and plasmids

Strains or plasmids	Description	Source
<i>Escherichia coli</i>		
JM 107	SupE Δ(<i>lac-proAB</i>) <i>hsdR4</i> F' <i>traD36 proAB⁺ lacI^q</i> <i>lacZΔM15</i>	
M 109	JM 107 <i>recA</i>	
col1K	JM 107 <i>wcaJ</i> ::Kan ^r	this work
col 1K, DE3	col1K, DE3	this work
<i>Plasmids</i>		
pSM9711	plasmid pET-11a <i>fucT</i> , <i>Amp^r</i>	this work
pUC19	<i>Amp^r</i>	Biolabs
pFUCT1	pUC19 <i>fucT</i> , <i>Amp^r</i>	this work
pBBR	plasmid pBBR-MCS1, <i>Cm^r</i>	
	plasmid pBBR-MCS3, <i>Tet^r</i>	
pBBlgTB	plasmid pBBR-MCS1 <i>lgtB</i> , <i>Cm^r</i>	
pCW lgtA	plasmid pCW ori ⁺ <i>lgtA</i> , <i>Amp^r</i>	
pLNT1T	plasmid pBBR-MCS3 <i>lgtA</i> , <i>lgtB</i> , <i>Tet^r</i>	this work
pRC1C	pBBR-MCS1 <i>rcsA</i> , <i>Cm^r</i>	this work
pLNTR1C	plasmid pBBR-MCS1 <i>lgtA</i> , <i>lgtB</i> , <i>rcsA</i> , <i>Cm^r</i>	this work
pLNTR1T	plasmid pBBR-MCS3 <i>lgtA</i> , <i>lgtB</i> , <i>rcsA</i> , <i>Tet^r</i>	this work
Le ^X cells	strain col 1K, DE3 pLNTR1T, pSM9711	this work

Fucosyltransferase assay

Cells were suspended in 50 mM Tris-HCl buffer (pH 8.0) and disrupted with a French Press. Soluble extract (5 μL) was incubated with 20 μM GDP-fucose, 833 Bq ¹⁴C-fucose (Amersham), 5 mM LNT, 5 mM MnCl₂, 1 mM ATP, 50 mM Tris-HCl buffer (pH 7.2) in a final volume of 25 μL. The reaction mixture was incubated at 37°C for up to 2 hours. Reaction was stopped by addition of 500 μl of mixed bed resin (AG 1 × 8 in water, 1 : 4 p/v). The mixture was spun briefly, and incorporated fucose in the supernatant was quantified. Protein concentration was determined using the *Protein assay* kit (Biorad).

Quantification of oligosaccharides during fermentation

Fresh culture samples (1 ml) were centrifuged in microfuge tubes (2 min, 10,000 × g). The supernatants were saved for quantification of extracellular oligosaccharides. The pellets were resuspended in 1 ml of distilled water, boiled for 10 min and centrifuged as above. The second supernatant was kept to quantify the intracellular oligosaccharides.

After acid hydrolysis *N*-acetylglucosamine (HexN) content was quantified colorimetrically using the Ehrlich reagent [20]. Total fucose was quantified colorimetrically after acid hydrolysis by the cystein chloride reagent method [21]. Lactose concentration was quantified using the kit from Roche Diagnostic for the enzymatically determination of lactose and glucose.

Purification of oligosaccharides

At the end of the fermentation time course, the bacterial cells were recovered by centrifugation (20 min at 7000 g). The supernatants were saved for purification of extracellular oligosaccharides. The pellets were resuspended in a volume of distilled water equal to that of the original culture medium and the cells were permeabilized autoclaving at 100°C for 30 min. After another centrifugation (30 min at 7000 g) the cells debris were discarded and the supernatant was retained to purify the intracellular oligosaccharides.

Oligosaccharides were adsorbed on activated charcoal and, after thorough washing with distilled water, eluted as previously described with 50% (v/v) aqueous ethanol.

Oligosaccharides were further purified by size exclusion chromatography (SEC) on a Biogel P2 (4.5 × 95 cm) column, with distilled water as mobile phase, at a flow rate of 40 ml h⁻¹.

Oligosaccharides **1** and **2** were purified from enriched SEC fractions by a second chromatography on Biogel P2.

Oligosaccharides **3**, **4**, and **5** were purified from enriched SEC fractions by HPLC using a preparative amino column (Hypersyl 8 μ, Interchrom, 250 × 27 mm) coupled to a refractive index detector and eluted with a mixture of acetonitrile/water (65 : 35 for compound **3**; 70 : 30 for **4** and **5**) at a flow rate of 9 ml/min.

The purity of oligosaccharides was estimated by TLC plate analysis on silica gel eluted with the mixture butanol/acetic acid/water (2:1:1, two runs). Sugars were detected by dipping in orcinol sulfuric acid reagent (sulfuric acid/ethanol/water 3:70:20, orcinol 1%) and heating.

Carbohydrate structural analyses

¹H-NMR spectra were recorded at 30°C with a 400 MHz Avance (Bruker) spectrometer using D₂O as solvent. Mass spectra in the FAB(+) mode were recorded with a Nermag R 10 10C spectrometer.

Oligosaccharides (5–10 mg) subjected to methylation (**3**, **4** and **5**) were first reduced with NaBD₄ (5 mg/500 µl water and 20 µl ammoniac 20%) overnight at room temperature. The reaction was stopped by addition of 100 µl of glacial acetic acid. Reduced compounds were desalted by gel permeation on a TKHW40F/50F (Interchrom) column (500 × 21.2 mm) coupled to a refractive index detector and eluted with water at a flow rate of 4 ml/min. Methylation was done according to Hakomori [22]. Part of the permethylated samples were kept for FAB-MS analysis. The rest was hydrolyzed in two steps, first in formic acid (90%, 1 h under reflux), then (after evaporation) in trifluoroacetic acid (2 M, 3 h under reflux). Partially methylated derivatives were reduced with NaBD₄ and then peracetylated with the mixture acetic anhydride/pyridine (1:1, 100°C, 1 h). After evaporation, partially methylated alditol acetates (PMAA) were dissolved in chloroform and analyzed in GC-MS on a capillary column SP 2380 (Supelco) for neutral derivatives, or an OV1 column (Spikhl) for GlcNAc derivatives.

Acknowledgments

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Functional and Biochemical Characterization of *Escherichia coli* Sugar Efflux Transporters*

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A family of bacterial transporters, the SET (sugar efflux transporter) family, has been recently reported (Liu, J. Y., Miller, P. F., Gosink, M., and Olson, E. R. (1999) *Mol. Microbiol.* 31, 1845–1851). In this study, the biochemical and cell biological properties of the three *Escherichia coli* members (SetA, SetB, and SetC) of the family are characterized. We show that both SetA and SetB can transport lactose and glucose. In addition, SetA has broad substrate specificity, with preferences for glucosides or galactosides with alkyl or aryl substituents. Consistent with the observed *in vitro* substrate specificities, strains that hyperexpress SetA or SetB are desensitized to lactose analogues as measured by induction of the *lac* operon. In addition, strains that hyperexpress SetA are resistant to the growth inhibitory sugar analogue *o*-nitrophenyl- β -D-thiogalactoside. Strains disrupted for any one or all of the *set* genes are viable and show no defects in lactose utilization nor increased sensitivity to inducers of the *lac* operon and nonmetabolizable sugar analogues. The data suggest that the *set* genes are either poorly expressed under normal laboratory growth conditions or are redundant with other cellular gene products.

The recently described bacterial SET (sugar efflux transporter) family of efflux pumps shares amino acid sequence similarity with the Major Facilitator Superfamily of transport proteins (1–3). Members of the Major Facilitator Superfamily perform diverse functions including the uptake of nutrients such as sugars and the secretion of noxious agents, including antibiotics (2, 3). The SET proteins were first identified in *Escherichia coli*, which encodes three members (SetA, SetB, and SetC) that share a high degree (at least 70%) of amino acid sequence similarity (1). Prior to this work, *setA*, *setB*, and *setC* were given the generic names *yabM*, *yeiO*, and *yicK*, respectively, for *E. coli* open reading frames of unknown function. It was shown that two of the proteins (SetA and SetB) could catalyze the secretion of lactose (1). Two additional members were identified as open reading frames in *Deinococcus radiodurans* and *Yersinia pestis*. A recent examination of the sequenced microbial genomes data base yielded six additional, more distantly related, proteins.¹ At present, the transport properties of these new family members have not been characterized. The SET proteins are not ubiquitously present in bac-

teria, suggesting an ecologically specialized role for this family of pumps.

Sugar efflux has been reported in many bacterial species (4, 5), including *E. coli* (6–9). It was shown that sugar efflux is an integral part of the metabolism of lactose in *E. coli* (7). In a strain constitutive for the *lac* operon, the addition of lactose led to the immediate and stoichiometric appearance of the products (glucose, galactose, and allolactose) of β -galactosidase action in the medium (6). Consistent with this hypothesis, mutants defective in the uptake of glucose and galactose grow poorly on lactose as the sole carbon source (7).

Physiological evidence supports the hypothesis that efflux systems are involved in the detoxification of nonmetabolizable sugars in *E. coli* (8–11). Methyl- α -glucoside (MG),² a competitive inhibitor of glucose utilization (12, 13), enters the cell mainly by the transporter for glucose and mannose, the products of the genes *ptsG* and *pstM*, respectively, and accumulates to high levels as both the phosphorylated and the unmodified forms (9, 13). When glucose is the sole carbon source, growth inhibition by MG is due to both decreased uptake of glucose and interference with the utilization of intracellular glucose-6-phosphate, the latter because of the accumulation of MG and MG-6-phosphate (9, 12). It was shown that both MG and MG-6-phosphate are secreted from the cell by an uncharacterized mechanism, the latter being first dephosphorylated before secretion (9, 11, 12).

Many nonmetabolizable lactose analogues such as isopropyl- β -D-thiogalactoside (IPTG) and methyl- β -D-thiogalactoside are growth inhibitory when lactose is the only carbon source (10). These compounds enter the cell through the lactose permease, the product of the *lacY* gene (14). To prevent the accumulation of IPTG and methyl- β -D-thiogalactoside, these sugar analogues are first acetylated by the LacA transacetylase and then secreted from the cell by an unknown transporter (8, 10). Reentry into the cell is prevented because the acetylated sugar analogues are not substrates for the permease (8).

In this report, a role for the SET proteins (SetA, SetB, and SetC) in the metabolism of lactose or the detoxification of nonmetabolizable sugar analogues is investigated. As we show in this study, the range of sugars that are efflux substrates for the *E. coli* SET proteins include selective monosaccharides and disaccharides, in addition to glycoside analogues such as IPTG. Because lactose and IPTG are both substrates for Set protein-catalyzed efflux, we also generated null mutations in *setA*, *setB*, and *setC* and used these to help define the role of the Set proteins in *E. coli* sugar metabolism.

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¹ M. Saier, manuscript in preparation.

² The abbreviations used are: MG, methyl- α -glucoside; IPTG, isopropyl- β -D-thiogalactoside; ONPTG, *o*-nitrophenyl- β -D-thiogalactoside; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MOPS, 4-morpholinopropanesulfonic acid.

TABLE I
E. coli strains and plasmids

Strains	Relevant genotype or phenotype	Plasmid	Source or reference
W3110	F- λ -IN1 <i>rph-1 thyA36 deoC2</i>	none	K. Bertrand
MN102	W3110 Δ <i>acrAB</i>	none	K. Bertrand
MC4100	F- λ - <i>araD139</i> Δ (<i>argF-lac</i>)U169 <i>rpsL150 relA1 flbB5301</i>	none	T. Silhavy
ML308	<i>lacI</i>	none	ATCC 15224
JL172	ML 308 <i>setA::</i> Ω Sm ^r <i>setB::</i> Ω Cm ^r <i>setC::</i> Ω Tet ^r <i>zib-207::Tn10</i>	none	This study
JL186	W3110 <i>setA::</i> Ω Sm ^r <i>setB::</i> Ω Cm ^r	none	This study
B359	AB1157 <i>recD::Tn10</i>	none	Lab stock
Plasmid	Relevant properties	Source or reference	
pBAD 18	Amp ^r	33	
pBR322	Amp ^r , Tet ^r	32	
pBR-SetA, -SetB, or -SetC	Amp ^r <i>set</i> genes cloned into <i>NheI</i> - <i>Bam</i> HI site of pBR322	This study	
pBAD-SetA, -SetB, or -SetC	Amp ^r , <i>set</i> genes cloned into <i>SacI</i> - <i>Hind</i> III site of pBAD18	This study	

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Reagents—The bacterial strains and plasmids used in this study are listed in Table 1. Cultures were grown in L broth (10 g tryptone/liter, 5 g yeast extract/liter, 5 g NaCl/liter) at 37 °C unless otherwise indicated. Plates contained 15 g agar/liter and top agar contained 7.5 g agar/liter. Antibiotics and arabinose, purchased from Sigma, were added to the following concentrations: 50 μ g/ml ampicillin, 10 μ g/ml tetracycline, 12.5 μ g/ml chloramphenicol, 15 μ g/ml streptomycin, 35 μ g/ml kanamycin, 15 mM arabinose. M63 medium contained 3.0 g KH₂PO₄/liter, 7.0 g of K₂HPO₄/liter, 2.0 g (NH₄)₂SO₄/liter, 0.5 mg FeSO₄/liter, 1 mM MgSO₄/liter, 0.1 mg thiamine/liter, and 2.0 g glycerol/liter. Sugars, sugar analogues, hexokinase, glucose-6-phosphate dehydrogenase, CCCP, and NADP⁺ were purchased from Sigma. Ciprofloxacin was obtained from the Parke-Davis chemical collection. Radiolabeled lactose (55.0 mCi/mmol) was purchased from Amersham Pharmacia Biotech, and radiolabeled glucose (8.36 mCi/mmol) was purchased from NEN Life Science Products.

Construction of Plasmids—Standard DNA manipulation techniques were used (15). DNA corresponding to each of the *set* genes was obtained by the polymerase chain reaction. All polymerase chain reaction products were sequenced, and except for three silent mutations in *yabM*, the products were identical to the sequences reported in the EBI/GenBank™ data base. Construction of the plasmids pBAD-SetA, pBAD-SetB, and pBAD-SetC, previously referred to as pBAD-YabM, pBAD-YeiO, and pBAD-YicK (1), respectively, were described earlier (1). Plasmids pBR-SetA, pBR-SetB, and pBR-SetC are derivatives of plasmid pBR-LacY (1) where the *lacY* gene was replaced with the gene for either *setA*, *setB*, or *setC*, respectively.

Growth Curves—Overnight cultures of strains ML308/pBAD18, ML308/pBAD-SetA, and ML308/pBAD-SetB grown in L broth containing ampicillin were diluted 100-fold into the same medium or medium containing 15 mM arabinose. After 1.6 h of growth, ONPTG, dissolved at 20 times the final concentration in 60% dimethyl sulfoxide, or solvent only, was added to the indicated concentrations. The cultures were grown in a 96-well plate, and growth was followed with a SpectraMax 96-well plate reader (Molecular Devices Corp, Sunnyvale, CA).

Isolation of Inside-out Vesicles, Vesicle Transport Assays for [¹⁴C]Lactose and [¹⁴C]Glucose, Kinetics of SetA Catalyzed Lactose Transport, and Inhibitor Studies—Cells were grown in L broth with arabinose to an A₆₀₀ of 0.6–1.0 and harvested for inside-out vesicles, essentially as described previously (16). In the experiments described in Fig. 1 (A and C), the culture was grown in shake flasks. In the experiments described in Fig. 1 (B and D), the culture was grown in a fermentor with L broth containing ampicillin to an A₆₀₀ of 0.6–1.0. Subsequently, arabinose was added to 15 mM, and the culture was harvested 2 h later. Cell pellets were washed once with ice-cold lysis buffer (50 mM MOPS-KOH, pH 6.6, 180 mM NaCl, 10 mM EDTA), resuspended in lysis buffer to an A₆₀₀ of 40–80, and lysed in a French Press at 5000 p.s.i. The lysate was

centrifuged at 27,000 $\times$ g for 10 min to remove unlysed cells and debris. The supernatant was centrifuged for 1 h at 100,000 $\times$ g to pellet total membranes. Protein concentrations were determined by the Bradford protein assay kit (Bio-Rad) using bovine serum albumin as the standard. The transport assay was performed at 21 °C. A 1.5- μ l solution of 50 mM ATP, pH 7.0, 50 mM MgSO₄ was added to 13.5 μ l of a suspension of membrane vesicles (14.0 μ g protein/ml) followed by incubation at 21 °C for 30 s. Transport was initiated by the addition of 60 μ l of Buffer A (50 mM MOPS, pH 7.5, 10 mM MgSO₄, 192 mM NaCl) containing the labeled sugar ([¹⁴C]lactose: 0.181 mM, 55.0 mCi/mmol, or [¹⁴C]glucose: 0.57 mM, 8.36 mCi/mmol). At the specified times, the entire solution was mixed with 3 ml of ice-cold stop buffer (50 mM MOPS-KOH, pH 7.5, 180 mM LiCl) followed by filtration through Whatman glass fiber filters. The filters were washed twice with 3 ml of the same buffer, and radioactivity was measured in a liquid scintillation counter. For the CCCP-treated samples, CCCP was added to 80 μ M at the indicated time after the initiation of transport and allowed to incubate further before stopping the assay with stop buffer. For the kinetic studies of SetA catalyzed [¹⁴C]lactose transport, the stock [¹⁴C]lactose solution was diluted with unlabeled lactose so that the final concentrations in the assay were 0.33, 0.5, 1.0, 2.0, and 5.0 mM. In the assay for inhibitors of SetA catalyzed transport of [¹⁴C]lactose, Buffer A contained the tested inhibitor at 31.25 mM and [¹⁴C]lactose at 0.181 mM (55.0 mCi/mmol). The final concentrations of the test compound and lactose were 25 and 0.145 mM, respectively.

Induction of the lac Operon with IPTG and Lactose: β -Galactosidase Activity Assays—Log phase cultures (A₆₀₀ = 0.2–0.4) were grown at the indicated concentration of IPTG or lactose for 1 h. Subsequently, β -galactosidase activity in the culture was determined as described (17). To follow the kinetics of IPTG induction of the *lac* operon, IPTG was added to early log phase cultures (A₆₀₀ = 0.1) to final concentrations of 2.5 and 5.0 μ M. At the indicated times, samples of the culture were removed and assayed for β -galactosidase activity.

Gradient Plate Assay—Gradient plates were prepared by the method described previously (18). Antibiotics were used at the following concentrations: 15 μ g/ml kanamycin, 15 μ g/ml neomycin, 0.008 μ g/ml ciprofloxacin.

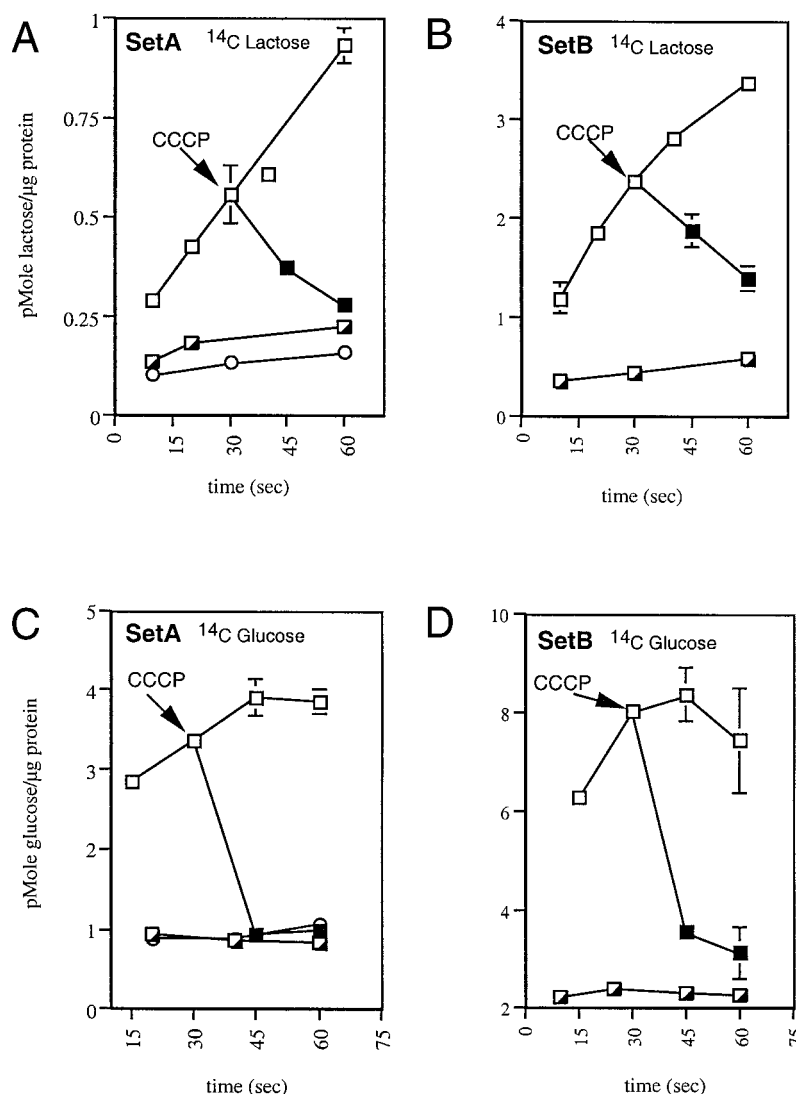
Lactose-dependent Glucose Efflux—The quantitative assay for glucose is essentially that described previously (6). Log phase cells growing in M63 with 0.2% glycerol (6) were pelleted and washed once with buffer containing 0.1 M NaPO₄, 1.0 mM MgSO₄, pH 7.6. Cells were resuspended in assay buffer (0.1 M NaPO₄, 7.0 mM MgSO₄, 6.0 mM ATP, 0.3 units/ml hexokinase, 2.5 μ g/ml glucose-6-phosphate dehydrogenase, 0.5 mg/ml NADP⁺, pH 7.6) to an A₆₀₀ of 0.25. The assay was initiated by the addition of lactose to 1.0 mM, and the absorbance at 340 nm was followed. To inhibit lactose uptake by LacY, cells were preincubated in assay buffer with CCCP at 60 μ M for 3.0 min before the addition of lactose. The glucose efflux activity was normalized to the β -galactosidase activity (17) in the solution.

Disruption of the set Genes and Construction of the Triple Disruption Strain—The disruption markers (19), referred to as interposons (denoted by the symbol Ω), which conferred resistance to streptomycin, chloramphenicol, or tetracycline, were inserted into *setA* (at *MscI*), *setB* (at *BsgI*), and *setC* (at *BsgI*), respectively, which were cloned in pBAD18. The disrupted alleles were individually integrated into the chromosome by one of two independent methods: 1) homologous recombination following transformation with linearized DNA into a *recD* strain (20) and 2) cloning of the disrupted alleles into the temperature sensitive replication plasmid pMAK705 (21) to allow use of a two-step method (21) to obtain double cross-over recombinants. The disrupted alleles were confirmed both by polymerase chain reaction analysis and by P1 mapping studies. The triple mutant was assembled in both the W3110 and ML308 strain backgrounds by P1 transduction (22).

RESULTS

A study of the transport properties of the SET proteins was undertaken as a first step toward elucidating the physiological function of this newly identified family of efflux proteins. Both *setA*, *setB*, and *setC* were cloned downstream from the arabinose-inducible promoter, generating the plasmids pBAD-SetA, pBAD-SetB, and pBAD-SetC, respectively, and the resulting plasmids transformed into *E. coli* strain MC4100. Inside-out membrane vesicles were prepared from cells that hyperexpressed the plasmid-encoded Set proteins. (In this configuration, an efflux pump would be expected to transport a radiolabeled substrate into the inside of the vesicle, which can be

FIG. 1. SetA and SetB both transport [^{14}C]lactose and [^{14}C]glucose. A and B, [^{14}C]lactose transport. C and D, [^{14}C]glucose transport. Inside-out membrane vesicles were prepared from strain MC4100/pBAD-SetA (A and C, $\square$, $\blacksquare$, and $\blacksquare$), strain MC4100/pBAD18 (A and C, $\circ$), and strain MC4100/pBAD-SetB (B and D) grown in the presence of arabinose. ATP was used to generate a proton gradient to energize transport. Partially filled square symbols ($\blacksquare$) represent transport in the absence of ATP. Uptake was initiated by the addition of radiolabeled lactose or glucose and was followed for 1 min. At the indicated time, CCCP was added to a final concentration of $80\ \mu\text{M}$ ($\blacksquare$). Each time point represents the mean of duplicate determinations $\pm$ range.



monitored as the accumulation of radioactivity in the vesicle interior.) It was shown previously and confirmed here that both SetA and SetB transport [^{14}C]lactose (Fig. 1, A and B). Transport was sensitive to the addition of the protonophore CCCP, which caused the release of the accumulated radioactivity to near basal levels. The accumulation of the labeled lactose was also dependent on the presence of either ATP or NADH, which gave equivalent rates of labeled lactose transport (data not shown) and was not observed in vesicles prepared from cells that harbored the control plasmid pBAD18. Transport activity in the SetB-containing vesicles was higher than that of SetA. The SetB and more recent preparations of SetA vesicles were prepared from cells grown with increased aeration. The transport activity of recent preparations of SetA vesicles is comparable with SetB and is likely due to increased SetA protein expression (data not shown).

The ability of SetA and SetB to transport [^{14}C]glucose was also tested. In this assay, a higher concentration of [^{14}C]glucose, which was of a lower specific activity than that of the [^{14}C]lactose, was used. Both SetA and SetB promoted the transport of [^{14}C]glucose, which was accumulated in the vesicle interior (Fig. 1, C and D). The accumulation of the radiolabeled glucose was also dependent on the presence of ATP, was sensitive to CCCP, and was not observed in vesicles prepared from cells that harbored the control plasmid pBAD18. We were unable to observe the transport of [^{14}C]galactose by SetA or

SetB (data not shown). In addition, vesicles prepared from the strain that harbored the *setC* expression plasmid was unable to transport any of the three sugars tested; however, the level of protein expressed was not determined.

Because our data from the SetA and SetB overexpression strains indicated that SetA has a broader substrate specificity than SetB (see below), an assay was developed to define the range of sugars and sugar analogues that could serve as SetA substrates. To facilitate the design of the assay, which was based on the inhibition of transport of radiolabeled lactose into inside-out vesicles, a study of the kinetic properties of lactose transport by SetA was conducted. Fig. 2A shows the kinetics of lactose transport at varying substrate concentrations. The rate of transport appears to be saturable (Fig. 2B) with an apparent K_m of $1.9\ \text{mM}$ and $V_{\max}$ of $0.12\ \text{pmol lactose}/\mu\text{g protein/s}$ (Fig. 2C). In the assay for possible substrates or inhibitors of lactose transport, the accumulation of radiolabeled lactose into inside-out vesicles was performed in the presence of unlabeled test compounds at $25\ \text{mM}$ and [^{14}C]lactose at $0.14\ \text{mM}$. (Note that this assay cannot distinguish between substrates that are effluxed by SetA and inhibitors that can compete with lactose for binding but that themselves are not transported.) The results of the competition assay are shown in Fig. 3 and are briefly summarized here. Of the tested sugars, trioses, tetroses, pentoses, and heptoses are poor inhibitors, whereas selective hexoses and disaccharides, the best being D-glucose (72% inhibi-

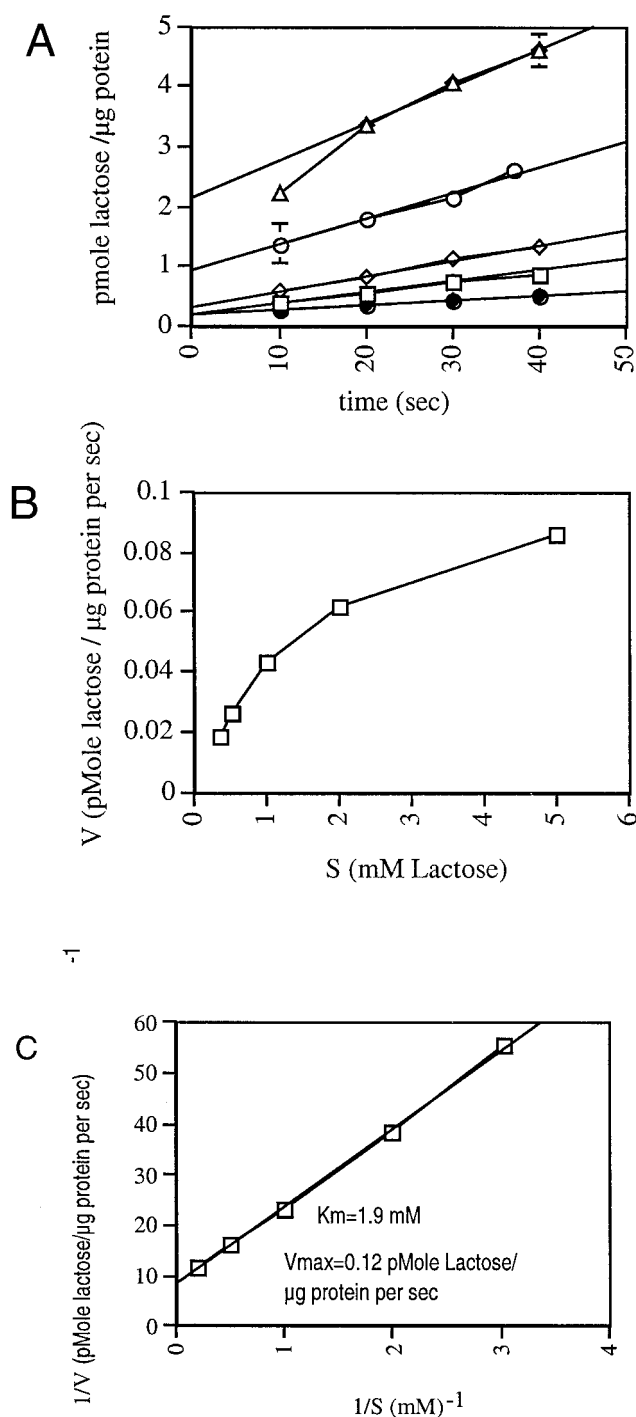


FIG. 2. Kinetic properties of SetA catalyzed [^{14}C]lactose transport. A, SetA-dependent accumulation of [^{14}C]lactose as a function of time. Inside-out membrane vesicles prepared from MC4100/pBAD-SetA were used for the transport studies. Transport was assayed at 0.33, 0.50, 1.0, 2.0, and 5.0 mM [^{14}C]lactose. Transport assayed with 0.33 mM [^{14}C]lactose in the absence of ATP is also shown. For clarity, the 5.0 mM substrate curve is omitted. Each time point represents the mean of duplicate determinations $\pm$ range. B, the relationship of the transport velocity versus substrate concentration. The slopes of the lines from A are plotted against the substrate concentration. C, Lineweaver-Burk plot of the data from A. $K_m = 1.9$ mM, $V_{\max} = 0.12$ pmol lactose/ μg protein/s. $\square$, 0.33 mM lactose; $\diamond$, 0.5 mM lactose; $\circ$, 1.0 mM lactose; $\triangle$, 2.0 mM lactose; $\bullet$, 0.33 mM lactose, no ATP.

tion; Fig. 3B) and cellobiose (88% inhibition; Fig. 3C), respectively, show inhibitory activity. Glucosides and galactosides with large alkyl or aryl aglycone substituents are the most potent inhibitors of lactose transport (91–100% inhibition;

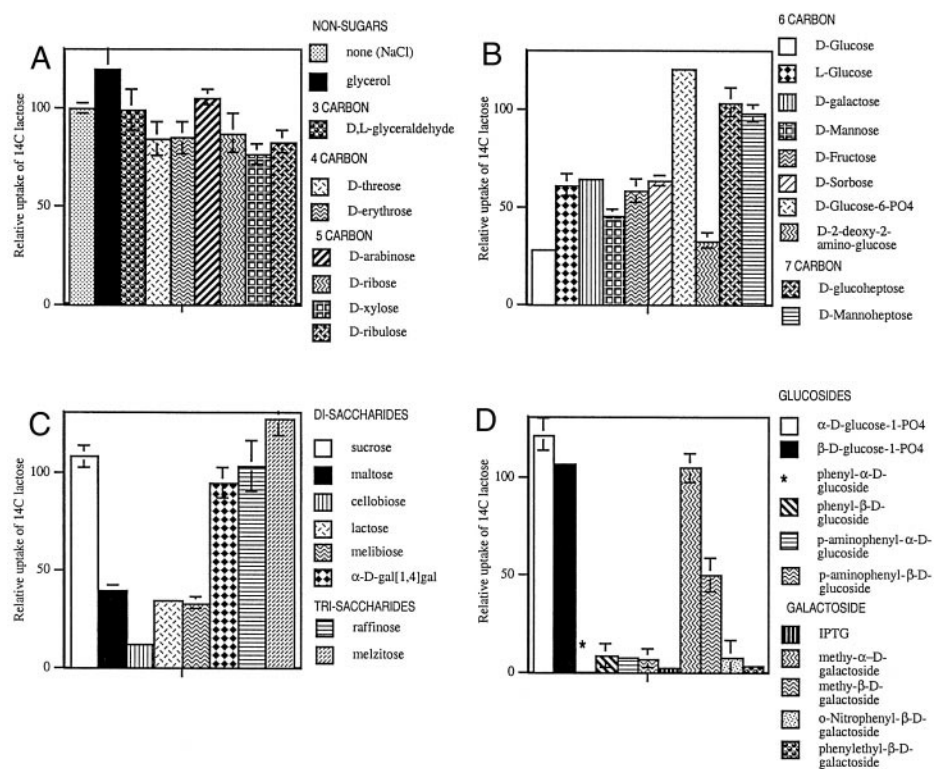
Fig. 3D). When the alkyl substituent is a small methyl group, such as that found in the methyl-galactosides, there is a significant reduction in the inhibitory activity (methyl- α -galactoside 0%; methyl- β -galactoside 50%) in addition to a pronounced preference for a β -linkage of the aglycone to the sugar (Fig. 3D). Interestingly, α - and β -D-glucose-1-phosphate are both inactive inhibitors. The inactivity is likely due to the presence of the negative charge and not a steric effect of the phosphate group (Fig. 3D). The first position can be replaced with the larger and uncharged phenyl substituent found in phenyl- α and phenyl- β -D-glucosides, which are both potent inhibitors (Fig. 3D).

A series of cell-based assays were designed to test whether SetA and SetB transport the substrates identified by the *in vitro* transport assays. A set of pBR322 derived plasmids were constructed that constitutively expressed each of the *set* genes by cloning them downstream from the promoter for the tetracycline resistance gene. The plasmids were transformed individually into *E. coli* strain W3110, which is wild type at the *lac* locus, and the resulting strains were each expected to hyper-express one of the Set proteins. These strains were tested for the ability of the individual Set proteins to transport either IPTG or lactose. Because IPTG and lactose are both inducers of the *lac* operon, the level of β -galactosidase activity should reflect the intracellular concentration of the inducer. If the inducer is effluxed from the cell by a Set protein, the level of β -galactosidase activity would be expected to be lower. Strains harboring individual *set* plasmids were titrated with either lactose or IPTG, and the level of β -galactosidase activity was measured. Fig. 4A shows that only SetA can efflux IPTG, because the level of β -galactosidase activity in the *setA*-containing plasmid was equal to that of the background at 0.1 mM IPTG. The level of β -galactosidase activity increased as the concentrations of IPTG rose from 1.0 to 100 mM, where it plateaued. This is the expected result if SetA is simply an efflux pump for the inducer. At high extracellular concentrations of the inducer, the rate of entry of the inducer exceeds the rate of efflux by SetA. In contrast, strains with either the *setB* or the *setC* plasmid, as well as the strain harboring the control plasmid pBR322, all showed high levels of β -galactosidase activity at 0.1 mM IPTG. The plateau level of β -galactosidase activity in the strain with the *setB* plasmid was lower than that of the control strain with pBR322, possibly reflecting the slow growth phenotype of this strain.

Fig. 4B shows that both SetA and SetB can efflux lactose. The β -galactosidase activity of the strain with the *setA* plasmid remained at background levels when titrated with lactose at up to 10 mM. Growth of this strain was not affected by the addition of lactose at the concentrations used. The β -galactosidase activity of the strain with the *setB* plasmid was equal to that of the background level at 0.1 mM lactose and was 22% of the level present in the control strain with pBR322 at 1 and 10 mM lactose. In contrast, the level of β -galactosidase activity in the strain with the *setC* plasmid was similar to that of the control strain with pBR322. Comparison of the lactose and IPTG titration curves indicates that IPTG is a better inducer of the *lac* operon, which is due to the fact that lactose is not active as an inducer until it is rearranged to allolactose by the action of β -galactosidase (23).

Because the *in vitro* transport studies indicated that aryl-glycosides may possibly serve as substrates for SetA, a cell-based assay was therefore designed to test this. It was previously reported that the intracellular accumulation of the toxic β -galactoside analogue, ONPTG, inhibits growth (24). Growth inhibition is dependent on the expression of *lacY*, because ONPTG is a substrate for the LacY permease (25). If ONPTG is also a substrate for SetA efflux, it would be expected that

FIG. 3. Inhibition of SetA catalyzed [^{14} C]lactose transport into inside-out vesicles. The assay measured the amount of SetA-dependent [^{14} C]lactose taken up into inside-out vesicles in 30 s in the presence of the indicated inhibitor at 25 mM. The amount of [^{14} C]lactose transported in the presence of 25 mM NaCl is taken to be 100. The classes of compounds tested for inhibitory activity were as follows: A, nonsugars, 3, 4, and 5 carbon monosaccharides. B, 6 and 7 carbon monosaccharides. C, disaccharides and trisaccharides. D, glucosides and galactosides. Each data point represents the mean of triplicate determinations $\pm$ S.D.



strains that hyperexpress SetA would be more resistant to the sugar analogue. A strain constitutive for the *lac* operon (ML308) was transformed with plasmids pBAD-SetA, pBAD-SetB or the control plasmid pBAD18. Transformants were tested for resistance to ONPTG by following the growth curves of cultures in the absence or presence of arabinose; the latter condition is expected to induce expression of SetA or SetB in the corresponding transformants. In the pBAD18 transformant, the presence of ONPTG caused a decrease in the growth rate (Fig. 5A). The severity of the growth inhibition was dependent upon the concentration of ONPTG in the medium. The presence of arabinose slightly decreased the growth yield (compare Fig. 5, A and B without ONPTG) but did not change the pattern of growth inhibition seen with increasing concentrations of ONPTG (Fig. 5B). Similarly, the pBAD-SetB transformant was as sensitive to ONPTG as the control pBAD18 transformed strain in medium with or without arabinose (Fig. 5, E and F). In contrast, the growth of the pBAD-SetA transformant was uninhibited in arabinose medium at ONPTG concentrations up to 1.71 mM (Fig. 5D). However, in the absence of arabinose, this strain was as sensitive to ONPTG as the pBAD18 transformant (Fig. 5C). These results indicate that ONPTG and likely other aryl- or alkyl- β -glycosides are substrates for SetA efflux.

It was shown above with the *in vitro* SetA lactose transport assay that positively charged sugars such as glucosamine and aminophenyl-glucosides are good inhibitors of lactose accumulation (Fig. 3, B and D). This feature is reminiscent of the aminoglycoside family of antibiotics such as kanamycin and neomycin. A cell-based assay was used to determine whether kanamycin and neomycin are substrates for SetA efflux, which would become apparent as resistance to the antibiotics. An *E. coli* strain deleted for *acrAB*, which encodes a multiple antibiotic efflux pump, was transformed with either plasmid pBAD-SetA or control plasmid pBAD18. The resulting transformants were tested on antibiotic gradient plates with or without arabinose. Again, arabinose is expected to induce expression of *setA*. In arabinose-containing medium, the strain with the *setA* plas-

mid was only slightly more resistant to kanamycin (31% growth across the gradient plate for pBAD-SetA versus 25% growth for pBAD18) and neomycin (63% growth for pBAD-SetA versus 53% growth for pBAD18) than the control strain with pBAD18 (Fig. 6). No difference in resistance to the tested antibiotics was observed in the absence of arabinose. As an additional control, both strains with either pBAD-SetA or pBAD18 showed identical sensitivity to the fluoroquinolone ciprofloxacin with or without arabinose in the medium. The data suggest that kanamycin and neomycin are poor substrates for SetA efflux.

It can be concluded from the *in vitro* and cell-based transport assays that SetA and SetB each can efflux glucose and lactose. Because the secretion of glucose was observed in the metabolism of lactose (6), the question emerged as to whether the *set* genes are involved in lactose utilization. To answer this question, all of the *set* genes (*setA*, *setB*, and *setC*) were disrupted in *E. coli* strains W3110 and ML308 by the insertion of different antibiotic resistance cassettes (see "Materials and Methods"). The mutant alleles were confirmed by polymerase chain reaction as well as by P1 mapping (data not shown). The growth rate of the triple mutant was identical to the parental strain in LB or minimal medium with lactose, glucose, glycerol, succinate, or lactate as the sole carbon source (data not shown). Also, the addition of lactose to log phase cells that were previously growing on glycerol did not cause any noticeable growth inhibition in the triple mutant compared with the parental strain. The triple mutant in the ML308 genetic background was further tested for glucose efflux in the presence of lactose. In this assay, washed log phase cells were placed in the coupled assay solution, which contained the enzymes hexokinase and glucose-6-phosphate dehydrogenase. The appearance of glucose was monitored as an increase in the absorbance at 340 nm because of the accumulation of NADPH as one of the final products of the sequential action of the two enzymes on glucose. In both the triple mutant and the parental strain, the addition of lactose led to the immediate appearance of glucose in the medium at a rate that reached steady state within 1.5 min

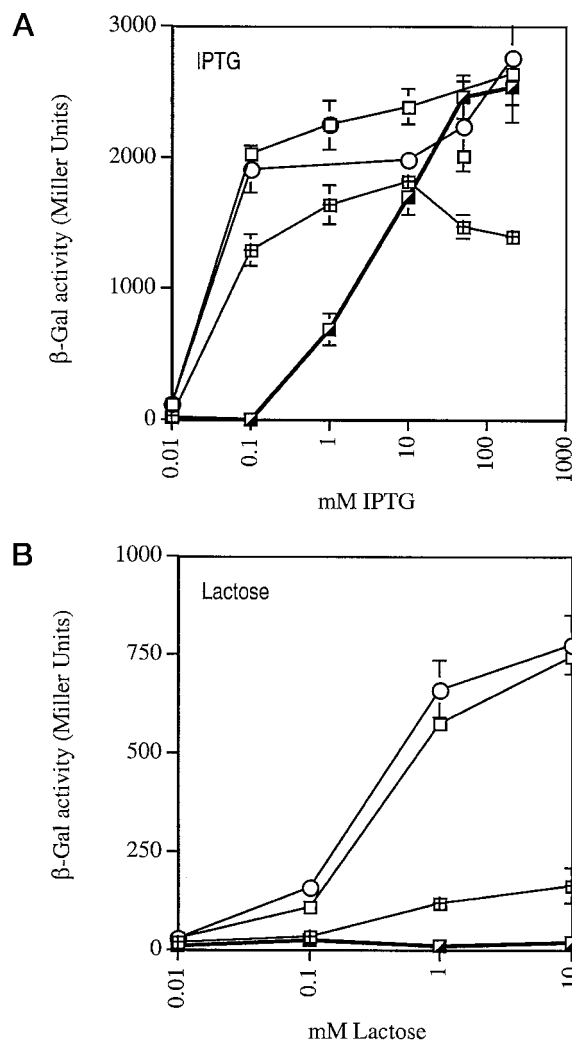


FIG. 4. Overexpression of SetA and SetB desensitize cells to inducers of the lactose operon. Log phase cultures of strains W3110/pBR322, W3110/pBR-SetA, W3110/pBR-SetB, and W3110/pBR-SetC were grown for one hour in the presence of the indicated concentrations of either IPTG (A) or lactose (B). Subsequently, the β -galactosidase activities (Miller units) in the cultures were determined. Each point represents the mean of duplicate determinations $\pm$ range. $\square$, pBR322; $\circ$, pBR-SetC; $\blacksquare$, pBR-SetB; $\blacktriangle$, pBR-SetA.

(data not shown). No difference in the steady state rate of glucose efflux between the triple mutant and parental strains was observed (Fig. 7). As a control to show that the detected glucose was due to its secretion from the cell and not to the action of free β -galactosidase in the medium on extracellular lactose, preincubation of both the triple mutant and parental strains with the protonophore CCCP at 60 μ M completely abolished the signal (Fig. 7). The complete inhibition of glucose efflux by treatment with CCCP indicated that there was no free β -galactosidase in the medium.

SetA and SetB can secrete inducers of the *lac* operon. The presence of efflux proteins for lactose or IPTG would be expected to lower the intracellular concentration of the inducer. To determine whether the triple mutant is more sensitive than the parental strain to induction of the *lac* operon by lactose or IPTG, the triple mutant in the W3110 strain background, which is wild type for the *lac* operon, along with the parental strain were titrated with IPTG or lactose and the levels of β -galactosidase activity in the cells after 1 h of exposure to the inducer was measured. No difference was observed between the triple mutant and the parental strain in this set of experiments (Figs. 8, A and B). Given that the *setA* hyperexpressing strain

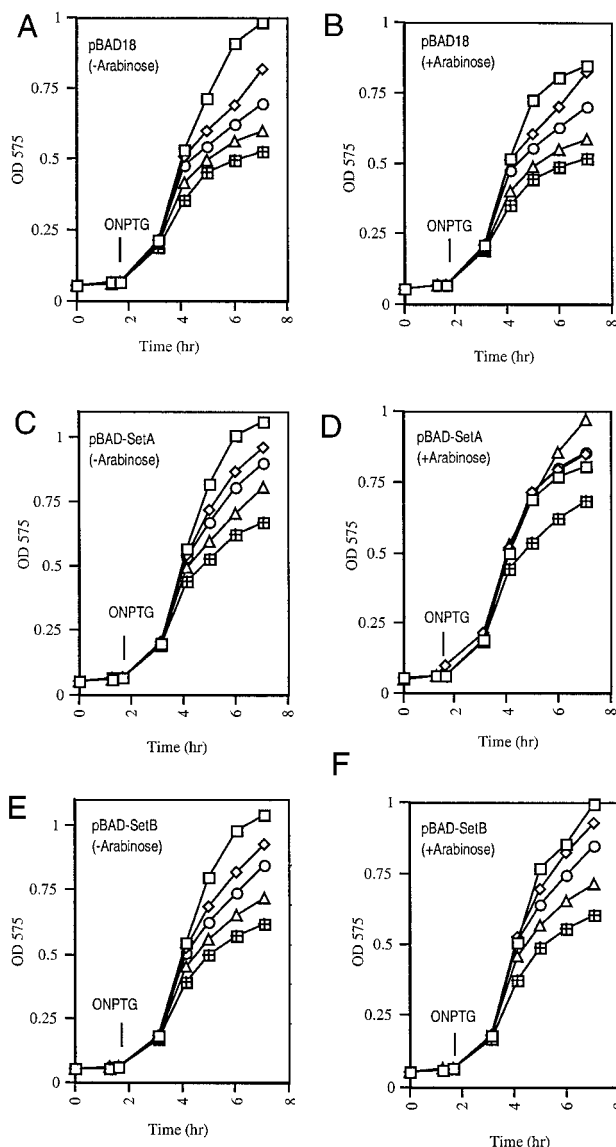


FIG. 5. Overexpression of SetA protects cells from the toxic sugar analogue ONPTG. Overnight cultures of strains ML308/pBAD18 (A and B), ML308/pBAD-SetA (C and D), and ML308/pBAD-SetB (E and F) grown in LB/ampicillin were diluted 100 fold into the same medium (A, C, and E) or medium with 15 mM arabinose (B, D, and F). After 1.6 h of growth, ONPTG was added to the indicated concentrations. The cultures were grown in a 96-well plate and monitored with a 96-well plate reader. $\square$, 0 mM ONPTG; $\diamond$, 0.42 mM ONPTG; $\circ$, 0.86 mM ONPTG; $\triangle$, 1.71 mM ONPTG; $\blacksquare$, 3.42 mM ONPTG.

does not respond at all to lactose and is desensitized to IPTG, this finding suggested that the level of SetA, and possibly SetB, is low in wild type cells. In an effort to employ a more sensitive assay for SetA activity, the kinetics of *lac* operon induction at low concentrations of IPTG (2.5 and 5.0 μ M) were examined in both the triple mutant and the parental strain. It was shown previously that induction of the *lac* operon, as measured by the level of β -galactosidase activity, is bi-phasic at low concentrations of IPTG (14). This is manifest as a lag in the rate of synthesis of β -galactosidase for 2–3 h before a high steady state rate is attained. This bi-phasic behavior is dependent on the *lacY* gene and can be explained as a positive feedback loop; leaky expression of the *lac* operon results in a low basal level of LacY permease activity, which catalyzes the uptake of the inducer IPTG, leading to higher rates of synthesis of the *lac* operon. If the SetA protein is present at an appreciable amount in the wild type cell, the triple mutant might be expected to

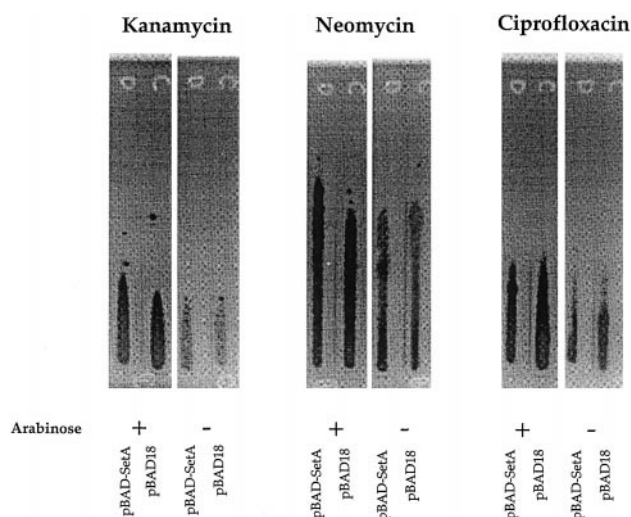


FIG. 6. **Hyperexpression of SetA confers low level resistance to aminoglycosides.** Resistance of strains MN102/pBAD18 and MN102/pBAD-SetA to the indicated antibiotic was determined by the gradient plate assay. Strains were tested on plates with or without arabinose. The maximum concentrations of the antibiotic in each plate were: 15 μ g/ml kanamycin, 15 μ g/ml neomycin, and 0.008 μ g/ml ciprofloxacin.

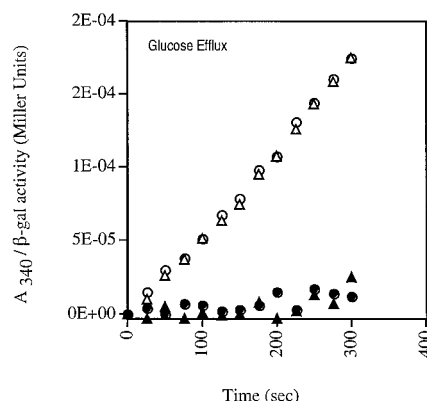


FIG. 7. **Lactose-dependent steady state glucose efflux in the triple *set* mutant and the parental strain.** Log phase cells of strains ML308 (parent) and JL172 (triple *set* mutant) were placed in assay buffer for glucose. Steady state glucose efflux was established at 1.5 min after the addition of lactose to 1 mM. The time and A_{340} at 1.5 min after lactose addition was arbitrarily set to 0 in the graph. The absorbance was normalized to the intracellular β -galactosidase activity (in Miller units). As control for extracellular β -galactosidase activity, cells were preincubated in assay buffer for 3 min in the presence of 60 μ M CCCP (filled symbols) before the addition of lactose. Δ , parent; $\blacktriangle$, parent with CCCP; $\circ$, triple mutant; $\bullet$, triple mutant with CCCP.

reach the high steady state rate of β -galactosidase synthesis sooner, because there would be no efflux protein to counteract the initial low levels of LacY. As shown in Fig. 8C, the kinetics of *lac* operon induction, with 2.5 and 5.0 μ M IPTG, is the same for both the triple mutant and the parental strain. This result further supports the hypothesis that the expression of *setA* is low in the wild type cell. This conclusion is also consistent with our observation that the triple mutant is not more sensitive than the parental strain to growth inhibition by toxic sugar analogues such as IPTG, ONPTG, or methyl- α -glucoside (data not shown).

DISCUSSION

Biochemical characterization of the newly discovered SET proteins showed that SetA and SetB can transport selective monosaccharides and disaccharides. Using an inside-out vesicle system, glucose and lactose were demonstrated to be substrates for SetA and SetB efflux. In addition, as judged by the

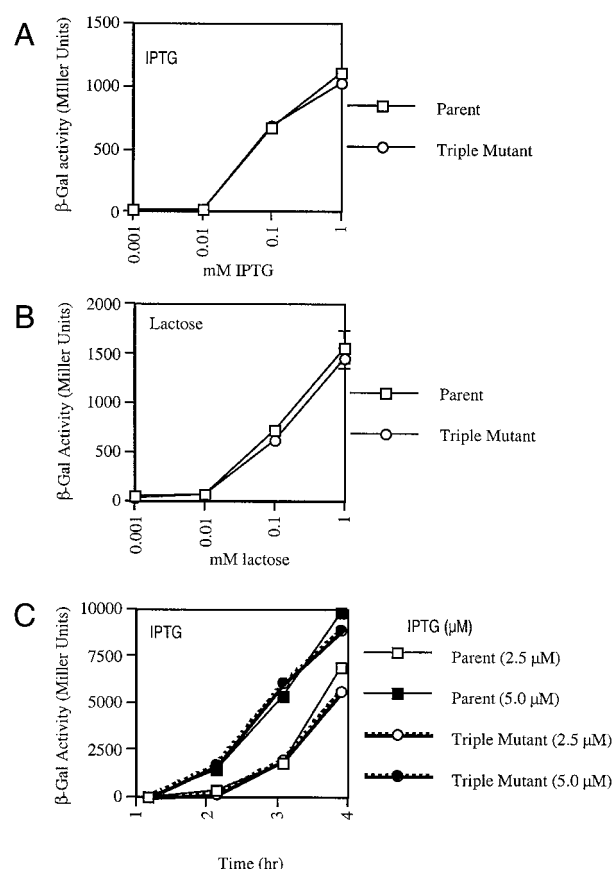


FIG. 8. **Sensitivity of the triple *set* mutant and the parental strain to induction of the *lac* operon with IPTG and lactose.** Log phase cultures of strains W3110 (parent) and JL186 (triple *set* mutant) were grown in the presence of the indicated concentration of IPTG (A) or lactose (B) for 1 h. Subsequently, the β -galactosidase activities (in Miller units) in the cultures were determined. C, time course of *lac* operon induction with 2.5 and 5.0 μ M IPTG. IPTG was added to log phase cultures of strains W3110 (parent) and JL186 (triple *set* mutant) to final concentrations of 2.5 and 5.0 μ M at time 0. The β -galactosidase activity (Miller Units) in the cultures was followed for 4 h. Each data point represents the mean of duplicate determinations $\pm$ range.

set of substrates considered here, SetA has broader substrate specificity than SetB. SetA also transports glucosides and galactosides with alkyl or aryl substituents. The results of these *in vitro* transport studies were confirmed using a series of cell-based assays for transport. Overexpression of SetA or SetB desensitized cells to the induction of the *lac* operon with lactose as the inducer but only SetA overexpression desensitized cells to IPTG. Transport of aryl- β -glycosides by SetA was confirmed by a growth sensitivity assay, because cells that overexpress SetA were more resistant to the toxic sugar analogue ONPTG. Although vesicles or cells with the *setC* expression constructs were unable to transport glucose, galactose, or lactose, the high degree of protein sequence identity of SetC to SetB (70%) suggests that the function is also conserved. It remains possible that *setC* may be poorly expressed in our plasmid constructs. However, each of the *set* genes, when present on a high copy number plasmid and expressed from the strong *lac* promoter, inhibited growth when induced with IPTG (data not shown), suggesting that a protein product was expressed in all cases. Additional transport studies with other sugars are warranted to identify substrates for SetC.

The range of substrates that are transported by SetA and SetB suggested possible roles for the SET proteins in lactose metabolism and in the secretion of nonmetabolizable sugar analogues. However, a mutant disrupted in all three *set* genes

showed no defect in lactose utilization; growth was normal in minimal medium supplemented with lactose as the sole carbon source, and glucose efflux was normal. In addition, no increase in sensitivity to the sugar analogues IPTG, methyl- β -D-thiogalactoside, and ONPTG was observed. These results indicate that other transporters, possibly one of the newly identified *E. coli* members of the SET family,¹ are responsible for the observed efflux of glucose in wild type cells. Interestingly, it was recently reported that the *E. coli* multidrug efflux pump *cmlA* may have IPTG efflux activity (26).

Our results suggest that the *set* genes are poorly expressed in *E. coli* under normal laboratory growth conditions. Consistent with this hypothesis, the triple *setABC* mutant has no observable phenotype. Because the expression of many bacterial efflux systems are induced by their substrates (3), it is likely that the true inducers or inducing conditions for the *set* genes have yet to be found. It is quite possible that the *set* genes function in a protective role, because hyperexpression of SetA was shown to protect cells against ONPTG. However, conditions that induce bacterial gene expression are frequently not obvious. Operons that were once thought to be poorly expressed (e.g. the β -glucoside operon (27, 28) and the chitose operon (29)) have been shown to be induced under conditions that were not apparent when these systems were first discovered. Indeed, methyl- β -glucoside and methyl- α -glucoside can modestly induce (about 4-fold) *setA* expression.³

The growth inhibitory effects of toxic sugar analogues may result from either the inhibition of uptake of a *bona fide* sugar substrate or the interference with normal metabolism by the intracellularly accumulated sugar analogues. The effectiveness of an efflux system to protect the cell from a toxic sugar analogue would be dependent on the rate of entry of the analogue into the cell and the activity of the efflux system. A sugar analogue that would otherwise be poisonous but have no pathway to enter the cell is harmless. As exemplified by ONPTG, sensitivity is dependent on the expression of *lacY*, the transporter for β -galactosides (24, 25). On the other hand, it would be difficult to protect a cell from a toxic sugar analogue that enters the cell through a highly efficient and constitutively expressed uptake system. It is likely that the physiological substrates for Set protein efflux would also be expected to be substrates for *E. coli* uptake systems. Under this scenario, the role of the Set proteins would be to secrete toxic sugar analogues that are taken up by mistake. However, it remains possible that the Set proteins function to remove toxic sugar-like by products that are produced by normal *E. coli* metabolism.

High level expression of SetA alone would be expected to be sufficient to protect cells from selective sugar analogues that are not modified upon entry. Among the known sugar transporters in bacteria, one family of transporters, the PTS (phosphoenolpyruvate-sugar phosphotransferase system) family, modifies the sugars upon entry by the addition of a phosphate group (30, 31). Our biochemical studies showed that negatively charged sugars are not substrates for SetA efflux. This is reminiscent of an unidentified transporter for the removal of MG (9, 11, 12). In the case of MG, entry into the cells is mainly through *ptsG* and *ptsM*, the PTS uptake proteins for glucose and mannose, respectively (13). MG accumulates in the cell predominately in the phosphorylated form as MG-6-phosphate (9). Presumably a phosphatase converts MG-6-phosphate to MG before secretion by the unidentified transporter. This detoxification system would rely on the unidentified phosphatase to discriminate between the phosphorylated sugar analogue

and the *bona fide* phosphorylated sugar, because only the uncharged sugar would be a substrate for efflux. We have not tested the *set* mutants for defects in MG secretion, although MG inhibits SetA catalyzed *in vitro* lactose transport (data not shown). However, the triple *set* mutant was not more sensitive to the growth inhibitory effect of MG as compared with the parental strain (data not shown).

The elucidation of the physiological function of the *set* genes would depend on finding growth conditions where the *set* mutants display a defective phenotype. Because the data suggest that the *set* genes are poorly expressed under normal laboratory growth conditions, a first step would be to identify conditions that induce *set* gene expression. Reporter strains have been constructed that carry chromosomal *set* fusions to *lacZ* or other reporter enzymes to screen for conditions or sugars that induce *set* gene expression. It may also be possible to design a genetic selection for chromosomal mutants that are up-regulated for *setA* expression based on its glycoside (such as IPTG) efflux activity.

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³ J. Y. Liu, unpublished observations.