

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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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: 22
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

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

Asunto: Radicación: 11-168961- -5
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 288
Folios: 22

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				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	No. 11-168961-00000-0000	07/Diciembre/2011
Reivindicaciones:	No. 11-168961-00000-0000	07/Diciembre/2011
Dibujos:	No. 11-168961-00000-0000	07/Diciembre/2011
Secuencias:	No. 11-168961-00000-0000	07/Diciembre/2011

2. 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. En el presente caso se estudiará la totalidad del capítulo reivindicatorio No. 11-168961-00000-0000 (reivindicaciones 1-17).

Título de la solicitud: “Síntesis de HMO”.

El problema planteado en esta solicitud consiste en la necesidad de proporcionar métodos de producción biotécnica de oligosacáridos para incrementar el rendimiento de

los oligosacáridos.

Para resolver este problema técnico la solicitud en estudio proporciona una célula que puede ser cultivada de manera estable en un medio, que ha sido modificada para producir oligosacáridos y un método para producir oligosacáridos donde se emplea la célula mencionada.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N 15/63, C12N 1/100

3. De la solicitud de patente

Descripción (Art 28 D 486)

La descripción no divulga la invención de manera suficientemente completa cualquier célula transformada para producir oligosacáridos para que el la persona del oficio normalmente versada en la materia pueda ejecutarla (ó reproducirla). La solicitud únicamente divulga un ejemplo en el que mediante una transformación de E.coli con setA, FucP, LacY y FutAco bajo condiciones particulares de cultivo resulta en una producción incrementada de 3-fucosilactosa. Es poco probable que la combinación de cualquier enzima involucrada en la síntesis de oligosacáridos y cualquier proteína de la familia de transportadores de efluentes azucarados, resulte en un efecto positivo. La intención de extender la enseñanza de E. coli a cualquier tipo de células parece irrazonable.

Debido a que la solicitud se refiere a material biológico, es necesario presentar el Certificado de Depósito para la cepa.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

- 1- La célula de la reivindicación 1 debe ser definida como una línea celular y debe incluir las modificaciones (definidas a partir de las secuencias), lo anterior para tener claras las características de la célula y debe estar sustentada con el número de depósito. La enzima involucrada en la síntesis de oligosacáridos y la proteína de los transportadores de efluentes azucarados deben estar definidas por su estructura y de acuerdo a lo sustentado en la descripción. La célula modificada reivindicada está definiendo la modificación por el resultado a alcanzar al referirse a una proteína que es codificada por la modificación realizada (Reivindicaciones 4, 8,9).
- 2- Las células fúngicas, animales y vegetales de la reivindicación 2 no están sustentadas en la descripción.
- 3- La expresión “una enzima que participa en la síntesis de oligosacáridos” no es clara y es muy general. Se sugiere definir las características de la invención por sus características estructurales. Adicionalmente, la expresión “homólogo funcional y derivado del mismo” no es clara (Reivindicación 1). La reivindicación 1 no es concisa, porque presenta una excesiva complejidad, lo cual dificulta determinar la extensión del objeto que se busca proteger.
- 4- La reivindicación 7 no es clara debido a que la expresión “una proteína que facilita o promueve la importación de eductos requeridos para la síntesis de oligosacáridos” se presta a interpretaciones. “Un educto” podría ser cualquier molécula que pueda ser transformada.
- 5- La reivindicación 10 no es clara porque se refiere a “una vía catabólica para mono-, di- o polisacáridos seleccionados, que está, al menos parcialmente inactivada”. El

término “parcialmente” no es limitativo, porque hace opcional a la característica y no limita el alcance de la reivindicación. No se puede definir con claridad cuáles de los miles mutantes están incluidos, porque pueden afectar una vía catabólica.

- 6- Las expresiones “suplementos básicos”, “eductos requeridos para la síntesis de oligosacáridos” y “causantes de actividad genética” no son claras (Reivindicación 14).
- 7- El método de la reivindicación 12 no es claro, debido a que no se define el medio, ni las condiciones apropiadas para producir oligosacáridos. Se le recuerda al solicitante que la reivindicación debe tener todos los elementos esenciales que definen la invención.
- 8- La reivindicación 16 está definida por sus parámetros, en donde se define que los oligosacáridos comprenden al menos tres subunidades y/o presentan un peso molecular de al menos 480 g/mol. Se sugiere definir la reivindicación por sus características esenciales, estructurales o funcionales.

Se requiere al solicitante hacer las aclaraciones necesarias.

4. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación internacional: Junio 08 de 2009, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	Ishihara et al., 1999. J Clin Invest. December 1; 104(11): 1621– 1629.	“Overexpression of monocarboxylate transporter and lactate dehydrogenase alters insulin secretory responses to pyruvate and lactate in β cells”	1999
D2	WO9911773	“Transgenic mammals which produce oligosaccharides in their milk ”	11/03/1999
D3	Condomine, 2000. J. Bacteriol. Vol. 182 No. 5 1340- 1345	“Characterization of SotA and SotB, Two <i>Erwinia chrysanthemi</i> Proteins Which Modify Isopropyl- β -D-Thiogalactopyranoside and Lactose Induction of the <i>Escherichia coli</i> lac Promoter”	2000

El documento D1¹ divulga da a conocer la sobreexpresión en células animales de la lactohidrogenasa, enzima que está involucrada en la síntesis de oligosacáridos, y de transportadores de carboxilo en membrana (Resumen).

El documento D2² divulga un método para modificar células para producir un oligosacárido de interés mediante la transformación de las células con secuencia de nucleótidos que codifican para una enzima involucrada en la síntesis de dicho oligosacárido (Reivindicación 1).

El documento D3³ divulga que las bombas de efluentes azucarados funcionan removiendo azúcares tóxicos del citoplasma (Resumen)

5. Examen de Patentabilidad (Art 14 D486)

1. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC409861/>

2. [http://worldwide.espacenet.com/publicationDetails/claims?](http://worldwide.espacenet.com/publicationDetails/claims?CC=WO&NR=9911773A1&KC=A1&FT=D&ND=&date=19990311&DB=&locale=en_EP)

CC=WO&NR=9911773A1&KC=A1&FT=D&ND=&date=19990311&DB=&locale=en_EP

3. <http://jb.asm.org/content/182/5/1340>

Novedad (Art 16 D486)

La reivindicación 1 consiste en *“una célula que puede ser cultivada de manera estable en un medio, que está modificada para producir oligosacáridos, donde la célula está transformada de manera tal que comprende al menos una secuencia de ácido nucleico que codifica una proteína que participa en la síntesis de los oligosacáridos, caracterizada porque la célula también está transformada de manera tal que comprende una secuencia de ácido nucleico que codifica una proteína de la familia de los transportadores que participan en la exportación de los azúcares, o un homólogo funciona o un derivado de ésta”*.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Célula modificada para producir oligosacáridos	Sobreexpresión en células animales de dos genes (Resumen)
Comprende al menos una secuencia de ácido nucleico que codifica una enzima que participa en la síntesis de los oligosacáridos	Lactodehidrogenasa que está involucrada en la síntesis de oligosacáridos (Resumen)
Secuencia de ácido nucleico que codifica una proteína de la familia de los transportadores, que participan en la exportación de los azúcares	Transportadores de carboxilo en membrana (Resumen)

Las características esenciales de la célula de la reivindicación 1 habían sido divulgadas previamente en el documento D1, el cual da a conocer la sobreexpresión en células animales de lactohidrogenasa, enzima que participa en la síntesis de oligosacáridos y transportadores de carboxilo en membrana (Resumen).

En consecuencia, la reivindicación 1 no es nueva o carece de novedad, según lo divulgado en el documento D1.

La reivindicación dependiente 2 no menciona alguna característica que haga nueva a la célula de la reivindicación 1, por lo cual se considera que no es nueva.

Nivel inventivo (Art18 D486)

La reivindicación 3 consiste en *“una célula que puede ser cultivada de manera estable en un medio, que está modificada para producir oligosacáridos (...)”*.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2	D3
Una célula transformada que puede ser cultivada de manera estable en un medio modificada para producir oligosacáridos	Célula para producir un oligosacárido de interés mediante la transformación de células	No menciona
Comprende una proteína de la familia de los transportadores que participan en la exportación de azúcares	No menciona	Divulga que bombas de efluentes azucarados probablemente funcionan removiendo azúcares tóxicos

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 3 porque divulga células para producir un oligosacárido de interés mediante la transformación de las células con una secuencia de nucleótidos que codifican para una enzima involucrada en la síntesis del oligosacárido (Resumen de D2) y los métodos para la producción de las células (Reivindicaciones 1-5).

La diferencia entre la invención, definida en la reivindicación 3 y la célula de D2 consiste en que las células son transformadas para comprender al menos una secuencia que codifica para una proteína de la familia de transportadores de efluentes azucarados, un homólogo funcional o un análogo del mismo.

El efecto que logra el incluir la transformación de la célula para la producir un oligosacárido de interés es la codificación de una proteína de la familia de transportadores de efluentes azucarados, un homólogo funcional o un análogo del mismo.

El problema técnico objetivo de esta solicitud consiste en la necesidad de obtener una célula alternativa a la ya conocida en D2, para producir un oligosacárido de interés que codifique una proteína de la familia de transportadores de efluentes azucarados, un homólogo funcional o un análogo del mismo.

Sin embargo, la utilización de un transportador de efluente azucarado para secretar del medio los oligosacáridos de interés, ya está divulgada en el documento D3 que da a conocer que el miembro de la familia de transportadores de efluentes azucarados SotA reduce la concentración intracelular de IPTG, lactosa y arabinosa (Resumen), que las bombas de efluentes azucarados probablemente funcionan removiendo azúcares tóxicos (Pág. 1345) y que la presencia de tres miembros de SET en E. coli puede indicar que la estrategia de desintoxicación es ampliamente usada por las bacterias (Pág. 1345).

En consecuencia, la persona del oficio normalmente versada en la materia, utilizaría el transportador de efluente azucarado para secretar del medio los oligosacáridos de interés de acuerdo a lo divulgado en D3 en las células del documento D2 para llegar así al objeto de las reivindicación 3 de la solicitud. Por lo cual se considera obvia.

Adicionalmente, es poco probable que la combinación de cualquier enzima involucrada en la síntesis de oligosacáridos y cualquier proteína de la familia de transportadores de efluentes azucarados, resulte en un efecto positivo. La intención de extender la enseñanza de E. coli a cualquier tipo de células parece irrazonable.

Las reivindicaciones 3-11 no cumplen con el requisito de nivel inventivo (Art. 18).

La reivindicación 12 comprende un “*método para producir la célula, para producir oligosacáridos (...)*”.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2	D3
Método para producir la célula transformada	Método para producir células transformadas (reivindicaciones 1-5)	No menciona
Comprende una célula transformada que puede ser cultivada de manera estable en un medio modificada para producir oligosacáridos	Célula para producir un oligosacárido de interés mediante la transformación de células (Resumen)	No menciona
Comprende una proteína de la familia de los transportadores que participan en la exportación de azúcares	No menciona	Divulga que bombas de efluentes azucarados probablemente funcionan removiendo azúcares tóxicos
Extraer los oligosacáridos del medio de cultivo	Extracción de oligosacáridos (Pág. 22)	No menciona

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 12 porque divulga células para producir un oligosacárido de interés

mediante la transformación de las células con una secuencia de nucleótidos que codifican para una enzima involucrada en la síntesis del oligosacárido (Resumen de D2) y los métodos para la producción de las células (Reivindicaciones 1-5).

La diferencia entre la invención, definida en la reivindicación 12 y el método de D2 consiste en que las células son transformadas para comprender al menos una secuencia que codifica para una proteína de la familia de transportadores de efluentes azucarados, un homólogo funcional o un análogo del mismo.

El efecto que logra el incluir la transformación de la célula para la producir un oligosacárido de interés es la codificación de una proteína de la familia de transportadores de efluentes azucarados, un homólogo funcional o un análogo del mismo.

El problema técnico objetivo de esta solicitud consiste en la necesidad de obtener un método alternativo al ya conocido en D2, para producir un oligosacárido de interés que codifique una proteína de la familia de transportadores de efluentes azucarados, un homólogo funcional o un análogo del mismo.

Sin embargo, la utilización de un transportador de efluente azucarado para secretar del medio los oligosacáridos de interés, ya está divulgada en el documento D3 que da a conocer que el miembro de la familia de transportadores de efluentes azucarados SotA reduce la concentración intracelular de IPTG, lactosa y arabinosa (Resumen), que las bombas de efluentes azucarados probablemente funcionan removiendo azúcares tóxicos (Pág. 1345) y que la presencia de tres miembros de SET en E. coli puede indicar que la estrategia de desintoxicación es ampliamente usada por las bacterias (Pág. 1345).

En consecuencia, la persona del oficio normalmente versada en la materia, utilizaría el transportador de efluente azucarado para secretar del medio los oligosacáridos de interés de acuerdo a lo divulgado en D3 en el método del documento D2 para llegar así al objeto de la reivindicación 12 de la solicitud. Por lo cual se considera obvia.

Adicionalmente, es poco probable que la combinación de cualquier enzima involucrada en la síntesis de oligosacáridos y cualquier proteína de la familia de transportadores de efluentes azucarados, resulte en un efecto positivo. La intención de extender la enseñanza de E. coli a cualquier tipo de células parece irrazonable.

Las reivindicaciones 13-17 no cumplen con el requisito de nivel inventivo (Art. 18).

6. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Ishihara et al., 1999. J Clin Invest. December 1; 104(11): 1621–1629.	1 - 2	Novedad
D2	WO9911773	3 – 17	Nivel inventivo
D3	Condomine, 2000. J. Bacteriol. Vol. 182 No. 5 1340-1345	3 – 17	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.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-01-21

Elaboró: Lina Maria Lizaraso Trespalacios
Revisó: Jeny Paola Villate Becerra

Overexpression of monocarboxylate transporter and lactate dehydrogenase alters insulin secretory responses to pyruvate and lactate in β cells

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Previous investigations revealed low activities of lactate dehydrogenase (LDH) and plasma membrane monocarboxylate transporters (MCT) in the pancreatic β cell. In this study the significance of these characteristics was explored by overexpressing type A LDH (LDH-A) and/or type 1 MCT (MCT-1) in the clonal INS-1 β cells and isolated rat islets. Inducible overexpression of LDH-A resulted in an 87-fold increase in LDH activity in INS-1 cells. Adenovirus-mediated overexpression of MCT-1 increased lactate transport activity 3.7-fold in INS-1 cells. Although overexpression of LDH-A, and/or MCT-1 did not affect glucose-stimulated insulin secretion, LDH-A overexpression resulted in stimulation of insulin secretion even at a low lactate concentration with a concomitant increase in its oxidation in INS-1 cells regardless of MCT-1 co-overexpression. Adenovirus-mediated overexpression of MCT-1 caused an increase in pyruvate oxidation and conferred pyruvate-stimulated insulin release to isolated rat islets. Although lactate did not stimulate insulin secretion from control or MCT-1-overexpressing islets, co-overexpression of LDH-A and MCT-1 evoked lactate-stimulated insulin secretion with a concomitant increase in lactate oxidation in rat islets. These results suggest that low expression of MCT and LDH is requisite to the specificity of glucose in insulin secretion, protecting the organism from undesired hypoglycemic actions of pyruvate and lactate during exercise and other catabolic states.

J. Clin. Invest. **104**:1621–1629 (1999).

Introduction

Glucose homeostasis is precisely controlled in mammals. Its deterioration leads to diabetes mellitus, one of the most common diseases in the industrialized countries. Three major mechanisms contribute to maintain the glucose homeostasis: fine-tuning of insulin secretion by glucose from the pancreatic β cell, insulin-mediated glucose disposal into muscle and adipose tissue, and insulin-mediated suppression of glucose production from the liver (1). Insulin acts on its target cells by binding to specific receptors, which initiates an intracellular signaling cascade (2). On the other hand, the recognition of nutrients as stimuli by β cells is accomplished through metabolism-secretion coupling (3). This unusual mode of recognition of stimuli is dependent on the unique expression profiles of membrane transporters and metabolic enzymes. For example, the predominant isoform of hexokinase in β cells is glucokinase (hexokinase IV), whereas other cell types in general, except for hepatocytes, express hexokinase I or hexokinase II. In the β cell, where glucokinase is the flux-determining enzyme for glycolysis, glucose metabolism and insulin secretion are proportional to changes

in the blood-glucose concentration (4). High expression levels of the mitochondrial enzymes, pyruvate carboxylase (5, 6), and FAD-linked glycerol phosphate dehydrogenase (mGPDH) (7, 8), are also prominent features of β -cell gene expression.

Mitochondrial ATP generation plays a key role in the coupling of glucose metabolism to insulin secretion (3, 4). Glucose is metabolized to pyruvate by the glycolytic pathway. Pyruvate supplies carbons to the tricarboxylic acid (TCA) cycle, generating reducing equivalents. This leads to stimulation of the respiratory chain and ATP synthesis. Glucose also promotes transfer of electrons from cytosolic NADH to the respiratory chain through the glycerol phosphate and the malate-aspartate shuttles. The importance of these shuttles has recently been substantiated in islets from mGPDH-deficient mice treated with aminooxyacetate (AOA), an inhibitor of the malate-aspartate shuttle (9). Islets are comprised of 65 to 70% β cells (10). In islet non- β cells (mainly glucagon-producing α cells), the activity of lactate dehydrogenase (LDH) is several fold higher than in β cells (6, 8). This may determine the fate of glucose-derived pyruvate and would explain

why only as little as 15% of glucose carbons are oxidized in the mitochondria in islet non- β cells compared with about 90% in β cells (6).

In studies using whole rat or mouse islets, it has been observed that pyruvate cannot mimic the effect of glucose on insulin secretion, despite active metabolism (11–13). This has been referred to as the “pyruvate paradox.” Similar observations have been made for lactate (14, 15). Both pyruvate and lactate enter cells through the plasma membrane monocarboxylate transporter (MCT) (16). Recently, several MCTs have been cloned and characterized, and a family of at least 8 related cDNA sequences has been identified (17). They are abundantly expressed in several organs, such as skeletal muscle and brain, in which large quantities of lactate and pyruvate are released or taken up. The reported oxidation of pyruvate and lactate in whole islets may occur mainly in non- β cells, because these cells exhibit higher monocarboxylate transport activity than β cells (8). We postulate that pyruvate and lactate are not able to stimulate insulin secretion because of low expression of MCT and LDH in β cells.

The present study was designed to elucidate the pyruvate paradox, i.e., the failure of pyruvate and lactate to stimulate insulin secretion. To this end, we have explored the significance of low LDH and MCT activities in the β cell by overexpressing MCT-1 and LDH-A in rat insulinoma INS-1 cells and primary rat islets using both an inducible expression system and an adenoviral vector technique. Such gene manipulations affected neither glucose metabolism nor glucose-stimulated insulin secretion. Remarkably, increased MCT and LDH activities resulted in pyruvate- and lactate-stimulated insulin secretion in isolated rat islets.

Methods

Generation of INS-1 cells overexpressing rabbit LDH-A under the control of reverse tetracycline transactivator. The INS-r7 cell line (18), which carries the reverse tetracycline/doxycycline-dependent transactivator, was secondarily transfected with a plasmid harboring rabbit type A LDH (LDH-A) cDNA (a generous gift from Y. Briand; ref. 19). Six out of 40 hygromycin-resistant clones showed more than 30-fold overexpression of the LDH activity with maximal induction. Clone number 31, which exhibited highest LDH activity after the induction, was used for the present study and is termed as INS(LDH) cells. Another clone, number 29, has also shown similar results. INS(LDH) cells were cultured in RPMI-1640, as seen previously (20). Doxycycline (500 ng/mL) induction was started 36 hours before the experiments.

Isolation of rat islets. Islets were prepared from male Wistar rats (250–350 g) by collagenase digestion and handpicking. After 3–5-hour culture in RPMI, islets were infected with recombinant adenoviruses and further cultured for 24 hours.

Generation of recombinant adenoviruses bearing hamster MCT-1 cDNA (AdCAMCT-1) and rabbit LDH-A cDNA

(AdCALDH-A). Hamster MCT-1 cDNA (21) was obtained from American Type Culture Collection (No. 77499; Rockville, Maryland, USA). Construction of recombinant adenoviruses was as described previously (22). AdCALacZ, which express bacterial β -galactosidase, was used as a control adenovirus. INS(LDH) cells or islets were incubated with recombinant adenoviruses for 1 hour, washed once, and cultured in RPMI medium. INS(LDH) cells were infected 48 hours before experiments with recombinant adenoviruses at approximately 50 virus particles/cell, and islets were infected 24 hours before experiments at 2×10^5 virus particles/islet (50–70 virus particles/cell, assuming 3,000–4,000 cells/islet; ref. 10).

Immunoblot analysis. INS(LDH) cells were homogenized in hypotonic buffer (20 mM Tris [pH 7.4] 40 mM NaCl, 1 mM DTT, 1 mM PMSF). Cell homogenates were first centrifuged at 1,500 g for 15 minutes at 4°C. The supernatants were used for detection of LDH-A. Crude membrane preparations were then obtained by centrifugation of this supernatant at 200,000 g for 30 minutes at 4°C. For detection of MCT-1 or LDH-A expression in islets, islets were collected and directly

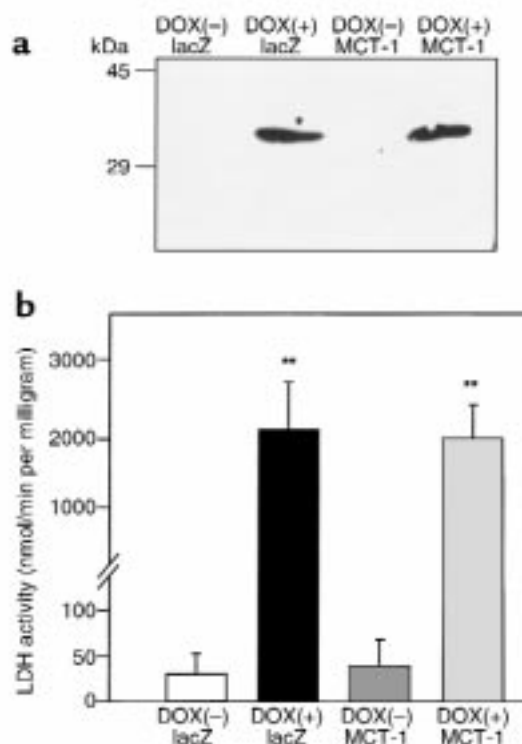


Figure 1

Doxycycline-inducible overexpression of LDH-A in INS-1 cells. (a) INS(LDH) cells were infected with either AdCALacZ or AdCAMCT-1 and cultured in the absence or presence of doxycycline (DOX; 500 ng/mL). Cellular homogenates (100 μ g) were dissolved in 11% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibody against purified rat liver LDH. (b) LDH activity was determined in cell homogenates using pyruvate as a substrate. Data show the mean $\pm$ SEM of 3 independent experiments. **Difference from control cells (noninduced and infected with AdCALacZ) at $P < 0.01$.

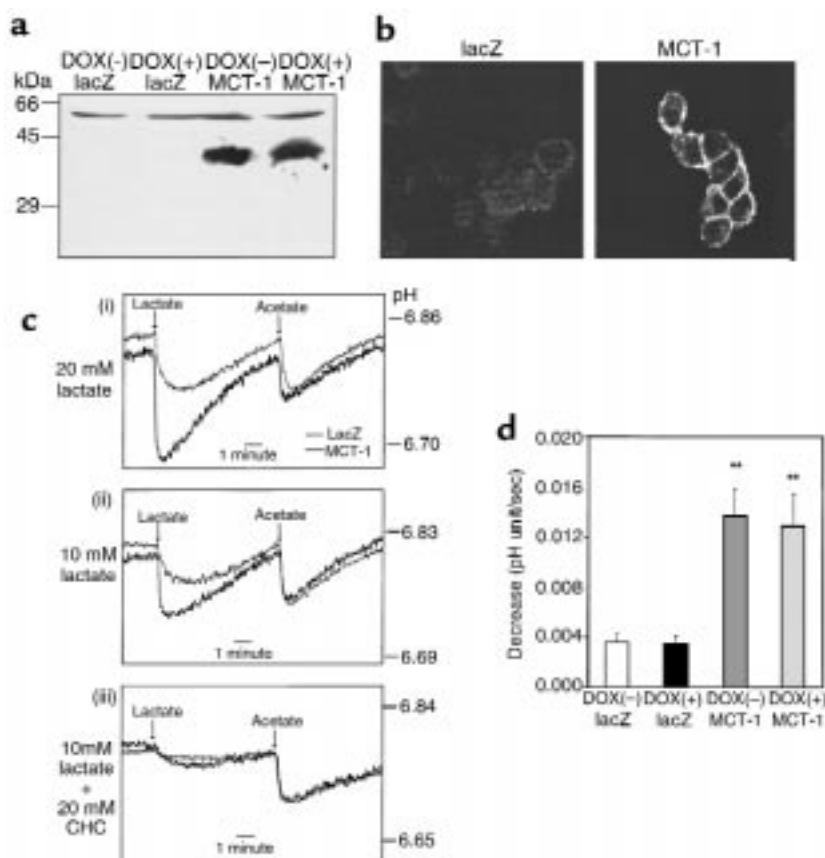


Figure 2

Overexpression of MCT-1 through a recombinant adenovirus in INS-1 cells. (a) INS(LDH) cells were treated as indicated in Figure 1a. Crude membrane preparations (50 μ g) were dissolved in 9% SDS-PAGE and immunoblotted with antibody against COOH-terminal peptide of rat MCT-1. (b) Infected INS(LDH) cells were fixed and immunostained with antibody against MCT-1. (c) Infected INS(LDH) cells were loaded with the pH sensitive dye BCECF and challenged with 20 mM lactate (i) or 10 mM lactate in the absence (ii) or presence (iii) of CHC (20 mM). Traces are representative of more than 4 traces from 2 to 5 experiments. (d) Initial rates of pH decrease were calculated from 4 traces each from 4 different cell preparations. **Difference from control cells at $P < 0.01$.

dissolved in SDS sample buffer after insulin secretion studies. Proteins were subjected to SDS-PAGE and were transferred to nitrocellulose membranes. Membranes were probed with chicken anti-rat MCT-1 antibody raised against the carboxy-terminal peptide (1:10000; ref. 23) or with rabbit anti-rat liver LDH (1:200, kindly supplied by A. Volkl; ref. 24) for 1 hour at room temperature, and then incubated for 1 hour with rabbit anti-chicken IgY (1:10,000) or with goat anti-rabbit IgG (1:3000) conjugated with horseradish peroxidase, respectively. Detection was accomplished with chemiluminescence (ECL; Amersham Switzerland, Zurich, Switzerland).

Measurement of LDH activity. Cells or islets were homogenized in 20 mM Tris (pH 7.4), 5 mM EDTA, 120 mM KCl, and 2 mM DTT. After centrifugation at 12,000 g for 10 minutes, supernatants (approximately 10 μ g protein) were incubated in a solution containing 80 mM Tris (pH 7.4), 5 mM EDTA, 2 mM pyruvate, and 20 μ M NADH. The reduction of NADH fluorescence (excitation: 340 nm; emission: 460 nm) was monitored using an LS-50B fluorometer (Perkin-Elmer, Beaconsfield, United Kingdom).

Measurement of lactate transport. INS(LDH) cells were harvested and cultured in a spinner culture flask for 2 hours. Cells (2×10^6) were suspended in a buffer (140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4 , 0.5 mM MgSO_4 , 1.5 mM CaCl_2 , 10 mM Hepes [pH 7.4]) and loaded for 30 minutes with 5 μ M of the acetoxymethyl ester of the pH-sensitive fluorescent dye 2',7'-bis(2-car-

boxylethyl)-5(6)-carboxyfluorescein (BCECF). Cells were placed in a cuvette in an LS-50B fluorometer, and BCECF fluorescence was measured at 530 nm (excitation at 500 nm) at room temperature (24–27°C). When needed, α -cyano-4-hydroxycinnamate (CHC) was added before starting the traces. Sodium salts of lactate, pyruvate, and acetate were used throughout the experiments. The fluorescence was calibrated to the pH value for each determination by adding HCl after permeabilization with 0.1% Triton X-100.

Measurement of insulin secretion. INS(LDH) cells (0.2×10^6 cells/well of 24-well plates) or islets (10 islets/tube) were incubated over a period of 60 minutes in Krebs-Ringer-bicarbonate-HEPES buffer (KRBH; 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4 , 0.5 mM MgSO_4 , 1.5 mM CaCl_2 , 2 mM NaHCO_3 , 10 mM HEPES [pH 7.4], 0.1% BSA) containing stimulators indicated. Insulin was detected by radioimmunoassay.

Measurement of mitochondrial oxidation. INS(LDH) cells (0.5×10^6 cells/35-mm dish) or islets (30 islets/tube) were transferred into a glass chamber and preincubated for 30 minutes in KRBH. Oxidation was initiated by replacing the buffer with 1 mL (INS cells) or 0.1 mL (islets) containing 0.2 μ Ci/chamber of [^{14}C]glucose, [^{14}C]pyruvate, or [^{14}C]lactate (DuPont-NEN, Boston, Massachusetts, USA). After 2-hour incubation at 37°C in the sealed chamber, 0.1 M HCl was added to cells and benzenethonium hydroxide (Fluka Chemie AG, Buchs, Switzerland) was injected into the bottom of the chamber to bind the liberated CO_2 . Following

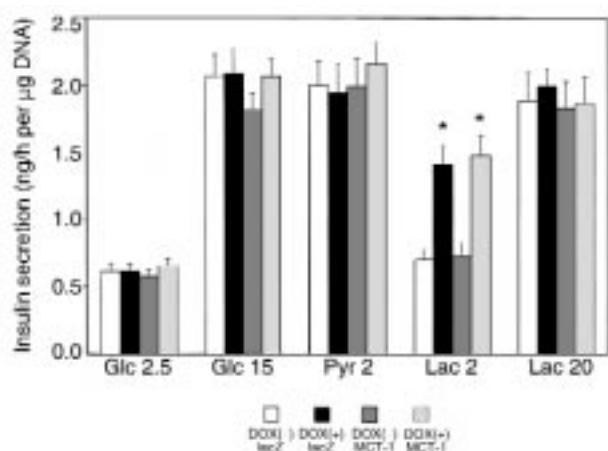


Figure 3

Insulin secretion from INS-1 cells overexpressing LDH-A and/or MCT-1. INS(LDH) cells cultured in the presence or absence of doxycycline and infected with either AdCALacZ or AdCAMCT-1 were incubated in KRBH with the indicated stimuli for 60 minutes. Insulin secreted in KRBH was quantified by radioimmunoassay and normalized by cellular DNA content. Data show the mean $\pm$ SEM of 4 or 5 independent experiments. *Difference from control cells (noninduced and infected with AdCALacZ) at $P < 0.05$.

an overnight incubation at room temperature, $^{14}\text{CO}_2$ production was counted in a LS6500 liquid scintillation counter (Beckman Instruments Inc., Palo Alto, California, USA).

Statistical analyses. Data are presented as mean $\pm$ SEM. Differences between groups are assessed by Student's *t* test for unpaired data.

Results

Overexpression of LDH-A and/or MCT-1 in INS-1 cells. INS-1 cells were stably transfected with an expression vector harboring LDH-A cDNA under the tetracycline-inducible promoter. One of the clones, designated INS(LDH), with the highest LDH-A expression after doxycycline induction, has been chosen for subsequent analyses. Doxycycline strongly induced the expression of a 35-kDa protein as detected by Western blot analysis probed with anti-liver LDH antibody (Figure 1a). Noninduced control cells do not express LDH-A, confirming the previous data that the endogenous LDH isoform in INS-1 cells is LDH-B₄ (8). In good agreement with the results of immunoblot analysis, nearly a 100-fold increase in LDH activity was found in doxycycline-treated cells (Figure 1b). LDH-A overexpression was not affected by MCT-1 co-overexpression (Figures 1a and 1b). Immunocytochemical and cell fractionation analyses have shown that overexpressed LDH-A was almost exclusively localized in the cytosol (data not shown).

To overexpress MCT-1 simultaneously with LDH-A, a recombinant adenovirus, AdCAMCT-1, was constructed. Immunoblot analysis probed with anti-MCT-1 antibody demonstrated that 42-kDa protein was abundantly expressed only in INS(LDH) cells infected with AdCAMCT-1 (Figure 2a). Induction of LDH-A

overexpression did not affect MCT-1 overexpression in INS(LDH) cells. Immunocytochemical analysis showed that overexpressed MCT-1 was correctly targeted to the plasma membrane (Figure 2b). Although the staining intensity was heterogeneous, essentially 100% of cells expressed the exogenous MCT-1. The MCT-1 expression was low in the control cells. So far, 8 MCT-related cDNA sequences have been identified (17). It remains to be established which of the isoforms are expressed in primary β cells or INS-1 cells.

To confirm functions of the expressed MCT-1, lactate transport activities were estimated by monitoring the transient acidification upon addition of lactate in control and MCT-1-overexpressing cells loaded with the pH-sensitive fluorescent dye, BCECF. This well-established method avoids the use of radiolabeled compounds (25). Typical traces of cells loaded with BCECF are shown in Figure 2c. Basal intracellular pH values were not different between control and MCT-1-overexpressing cells. Reduction of pH in response to 20 mM lactate was significantly greater in MCT-1-overexpressing cells compared with control cells. In contrast, the response to 10 mM acetate, which enters cells mainly by simple diffusion (16), was not altered (Figure 2c [i]). To verify that lactate enters the cells by MCTs, 10 mM lactate was tested in the absence (Figure 2c [ii]) and presence (Figure 2c [iii]) of 20 mM CHC, a competitive inhibitor of MCTs (16). CHC inhibited lactate-evoked acidification by 84% in control cells (from 2.2 ± 0.4 to 0.4 ± 0.2 pH unit $\times 10^{-3}$ /sec; $n = 4$, $P < 0.01$). CHC also reduced the effect of 10 mM lactate by 82% in MCT-1-overexpressing INS(LDH) cells (from 7.7 ± 0.4 to 1.2 ± 0.3 ; $n = 4$, $P < 0.01$). This demonstrates that increased rate of pH reduction in MCT-1-overexpressing cells is indeed due to overexpression of functional MCT-1. Initial pH decrease rates in response to 20 mM lactate were calculated to estimate the lactate transport activity (Figure 2d). It was found that cells infected with AdCAMCT-1 have a 3.7-fold greater lactate transport activity than cells infected with the control virus. The increase in the lactate transport activity was independent of LDH co-overexpression and was also evident at 2 mM lactate (data not shown). Pyruvate (10 mM) transport activity was also increased after MCT-1 overexpression (2.1 ± 0.4 vs. 4.4 ± 0.9 pH unit $\times 10^{-3}$ /sec in control and MCT-1-overexpressing INS(LDH) cells; $n = 4$, $P < 0.05$).

Effects of LDH-A and/or MCT-1 overexpression on insulin secretion from INS(LDH) cells. Insulin secretion in response to several secretagogues was assayed in cells treated with doxycycline and/or AdCAMCT-1 (Figure 3). In all 4 types of cell preparations, the stimulatory glucose concentration (15 mM) induced more than 3-fold increases in insulin secretion, and there were no differences among the 4 cell preparations. Pyruvate is a potent stimulus for insulin secretion in INS-1 cells (8). Overexpression of MCT-1 and/or LDH-A, however, did not affect insulin secretion evoked by pyruvate (2 mM). In noninduced INS(LDH) cells, 2 mM lactate did not elicit insulin secretion. Remarkably, this concentration

of lactate was able to stimulate insulin secretion (2-fold) after the overexpression of LDH-A in INS-1 cells. In contrast, 20 mM lactate stimulated insulin secretion both in control and LDH-A overexpressing INS-1 cells. These effects were not altered by co-overexpression of MCT-1 (Figure 3). At 10 mM lactate, the incremental insulin secretion over the basal level was 0.32 ± 0.07 ng/h per micrograms DNA in control cells ($n = 3$, NS), which rose to 1.11 ± 0.13 after overexpression of LDH-A ($n = 3$, $P < 0.05$).

To gain insight into the mechanism underlying lactate-evoked insulin secretion in INS-1 cells, the metabolic consequences of LDH-A and/or MCT-1 overexpression in INS(LDH) cells were studied. The rate of [U - ^{14}C]glucose (15 mM) oxidation was not altered by overexpression of LDH-A and/or MCT-1 (Figure 4a). Importantly, the [U - ^{14}C]lactate (2 mM) oxidation rate was doubled in LDH-A overexpressing cells, regardless of MCT-1 co-overexpression, compared with that in control cells (Figure 4b). This emphasizes the good correlation between mitochondrial substrate oxidation and insulin secretion. The results obtained with AOA, an inhibitor of the malate-aspartate shuttle, suggest the importance of this shuttle for the reoxidation of cytosolic NADH (Figure 4c). Thus, lactate-stimulated insulin secretion, which is associated with NADH production in the cytosol, was abolished by 0.25 mM AOA in INS(LDH) cells. Insulin release by pyruvate, not generating cytosolic NADH, was unaffected by AOA. Glucose-induced insulin secretion, on the other hand, was blocked by AOA (Figure 4c).

Engineering of pyruvate-stimulated insulin secretion in rat pancreatic islets. INS-1 cells deviate from native β cells in that pyruvate stimulates insulin secretion in the former but not latter cells. We hypothesize that this difference is due to low transport capacity for pyruvate in native β cells. The fact that a membrane-permeable analogue of pyruvate, methylpyruvate, is a potent secretagogue in isolated islets supports this idea (12, 13, 26). To test this hypothesis, we infected isolated rat islets with AdCAMCT-1 to increase the monocarboxylate transport activity in the β cell.

MCT-1 protein was not detected in islets infected with AdCALacZ, whereas islets infected with AdCAMCT-1 exhibited significant levels of expression of MCT-1 after 24-hour culture (Figure 5a). The insulin secretory response was then examined in these islets (Figure 5b). Insulin secretion in response to 15 mM glucose was not different between control islets and islets overexpressing MCT-1. Pyruvate (10 mM) failed to stimulate insulin secretion significantly in islets infected with AdCALacZ. After infection with AdCAMCT-1, pyruvate caused 3-fold greater insulin secretory response compared to that at the basal glucose (Figure 5b). To examine whether the enhanced pyruvate-stimulated insulin secretion was due to an increase in the pyruvate metabolism in islets, the rate of [1 - ^{14}C]pyruvate oxidation was measured. Pyruvate oxidation was increased by 2-fold in islets overexpressing MCT-1

compared with control islets (Figure 5c). On the other hand, overexpression of MCT-1 did not affect the rate of [U - ^{14}C]glucose oxidation.

Engineering of lactate-stimulated insulin secretion in rat pancreatic islets. Although rat islets became responsive to pyruvate after overexpression of MCT-1, lactate did not elicit insulin secretion from islets overexpressing MCT-1 (Figure 5b). The enhanced lactate-stimulated insulin secretion after overexpression of LDH-A in INS-1 cells suggests that an efficient conversion of lactate to pyruvate is necessary for the

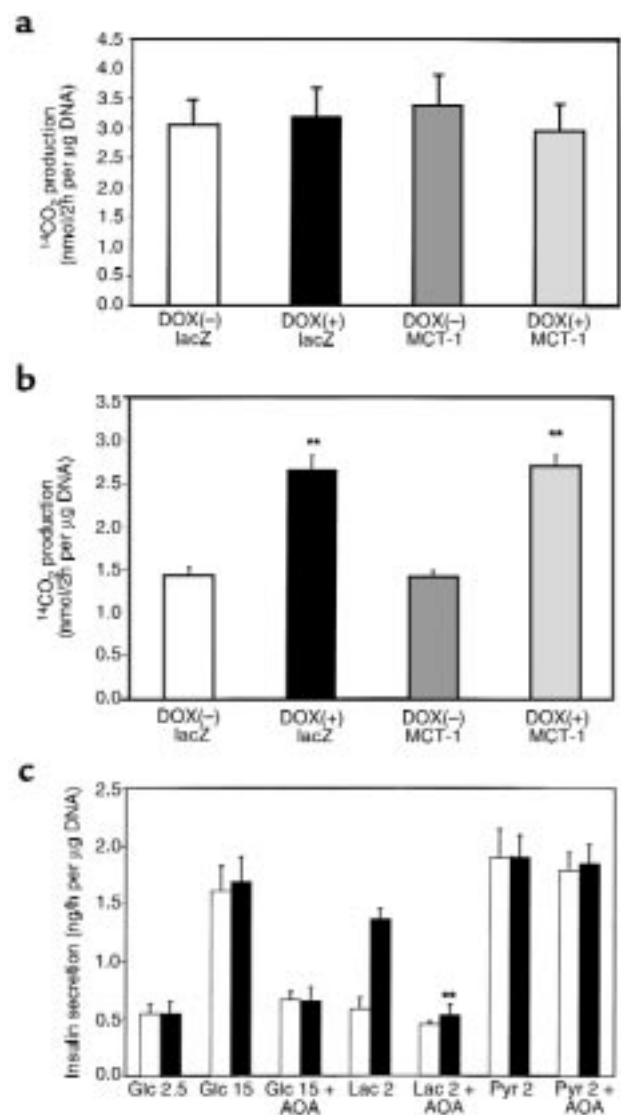


Figure 4

Mitochondrial oxidation in INS(LDH) cells overexpressing LDH-A and/or MCT-1. $^{14}CO_2$ formation during 2-hour incubation with 15 mM [U - ^{14}C]glucose (a) or 2 mM [U - ^{14}C]lactate (b) were measured. Data show the mean $\pm$ SEM of 3 independent experiments. **Difference from control cells (noninduced and infected with AdCALacZ) at $P < 0.01$. (c) INS(LDH) cells cultured in the presence (filled bars) or absence (open bars) of doxycycline were incubated in KRBH with the indicated stimuli. AOA (0.25 mM), an inhibitor of the malate-aspartate shuttle, was included where indicated. Statistical significance was obtained ($P < 0.01$) in lactate-stimulated insulin secretion between AOA-treated and nontreated cells overexpressing LDH-A.

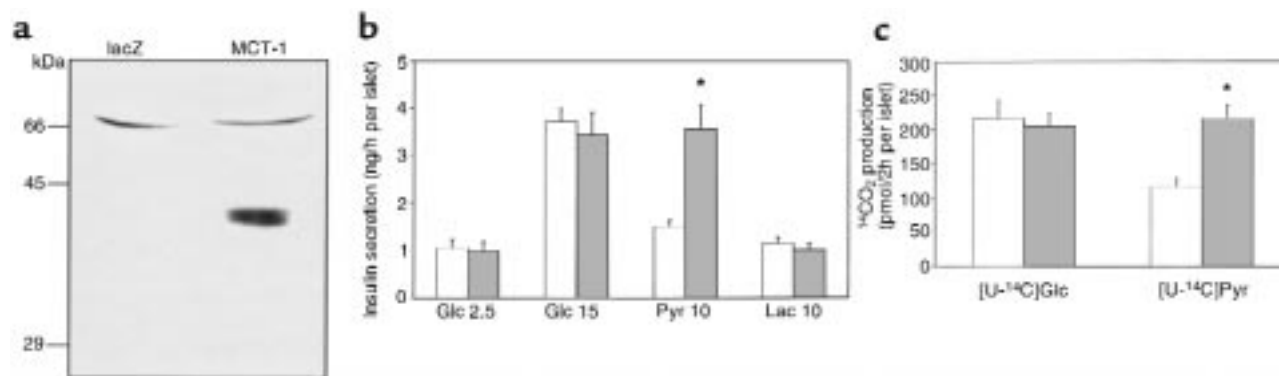


Figure 5

Overexpression of MCT-1 through a recombinant adenovirus and its effects on isolated islets. (a) One hundred twenty islets infected with either AdCALacZ or AdCAMCT-1 were used for immunoblot analyses. The result is representative of 3 independent experiments. (b) Ten islets infected with recombinant adenoviruses in each tube were incubated in KRBH with indicated stimuli for 60 minutes. Data show the mean $\pm$ SEM of 5 independent experiments. Statistical significance between MCT-1-overexpressing islets and control islets was obtained at 10 mM pyruvate ($P < 0.05$). (c) $^{14}\text{CO}_2$ formation during 2-hour incubation with 15 mM [U- ^{14}C]glucose or 10 mM [1- ^{14}C]pyruvate was measured. Data show the mean $\pm$ SEM of 5 independent experiments. *Difference from control islets at $P < 0.05$. Open bars, islets infected with AdCALacZ; shaded bars, islets infected with AdCAMCT-1.

stimulation by lactate. This was examined by overexpressing LDH-A simultaneously with MCT-1 in isolated rat islets.

Adenovirus-mediated introduction of LDH-A cDNA increased the amount of LDH-A protein by approximately 5-fold in rat islets (Figure 6a). The LDH activity was 7-fold greater in islets infected with AdCALDH-A alone or with both AdCALDH-A and AdCAMCT-1 (Figure 6b). Because the endogenous LDH activity is lower in β cells than in non- β cells (6, 8), the degree of LDH overexpression in β cells would be higher than that observed in whole islets. As was the case with INS-1 cells, insulin secretion evoked by glucose was not affected by overexpression of MCT-1 and LDH-A (Figure 6c). Although islets overexpressing LDH-A alone did not respond to pyruvate challenge, overexpression of both LDH-A and MCT-1 again produced pyruvate-stimulated insulin secretion. Interestingly, the same manipulation brought about marked (3.5-fold) stimulation by 10 mM lactate compared with control islets (Figure 6c). Lactate-induced insulin secretion was accompanied by a 3.7-fold enhancement of the [U- ^{14}C]lactate oxidation rate in rat islets overexpressing both LDH-A and MCT-1 (Figure 6d).

Discussion

Recent advances in the adenoviral vector technology have facilitated the transfer of multiple foreign genes into well-differentiated cell lines or primary cells, such as islet cells. To explore the significance of the documented low activities of LDH and MCT in the β cell relative to islet non- β cells (6, 8) and other cell types (8, 16), we have investigated the consequences of overexpression of LDH-A and MCT-1 on glucose-, pyruvate-, or lactate-stimulated insulin secretion in INS-1 cells and isolated rat islets. Because the chicken actin promoter used in our recombinant adenovirus constructs (27) is active in

both β cells and non- β cells (28), this approach diminishes differences in expression levels of LDH and MCT between these cells.

Pyruvate and lactate have been reported to be metabolized, but do not cause insulin secretion in pancreatic islets (11–15). Hence, the oxidation rate of 30 mM [1- ^{14}C]pyruvate was found to be as high as that of 16.7 mM [U- ^{14}C]glucose without any increase in insulin secretion (11). This dissociation between mitochondrial substrate oxidation and insulin secretion is known as the pyruvate paradox. It is most probable that the paradox could be explained by higher rates of pyruvate oxidation in the 30–35% non- β cells present in rat islets (10), which transport monocarboxylates 3 times more efficiently than β cells (8). This view is supported by results showing that islets from ob/ob mice, comprised of 90% β cells, oxidize 17 mM [U- ^{14}C]pyruvate 6 times less efficiently than 20 mM [U- ^{14}C]glucose (29). Also note that 5 mM pyruvate plus 3.3 mM glucose stimulates glucagon secretion but not insulin secretion in the perfused rat pancreas (30). The differences between pyruvate and glucose oxidation in ob/ob mouse islets may reside mainly in the much lower transport capacity for pyruvate compared with glucose across the β -cell plasma membrane. In contrast, pyruvate was reported to be avidly metabolized by mitochondria isolated from ob/ob mouse islets (31), indicating that the transport capacity of pyruvate across the inner mitochondrial membrane is not limiting. Our data demonstrating an increased pyruvate oxidation after overexpression of MCT-1 indicate that pyruvate transport by the plasma membrane MCT indeed is flux determining for pyruvate metabolism in isolated islets. Nonetheless, it cannot be excluded that the difference in efficiency between glucose and pyruvate metabolism could be determined not only by low expression of MCTs, but also by stimulation of mitochondrial shuttles (see below) by the former but not by the latter.

In INS-1 cells MCT activity is not rate limiting for the metabolism of pyruvate and lactate, because, first, pyruvate stimulates insulin secretion in these cells (Figure 3 and ref. 8), and second, lactate oxidation was not affected following overexpression of MCT-1. Nonetheless, MCT activity is markedly lower in INS-1 cells compared with that of RINm5F cells derived from the same rat insulinoma (8). The different potencies of pyruvate and lactate in INS-1 cells appear to be due to distinct metabolic fates of the 2 substrates. Because LDH-A but not MCT-1 overexpression led to insulin secretion at low lactate concentrations in INS-1 cells, initiation of secretion by lactate depends on pyruvate generation in the cell. This conclusion is supported by the results with AOA, which blocks reoxidation of NADH by the malate-aspartate shuttle (Figure 4c). The compound did not reduce pyruvate-stimulated insulin secretion while abolishing the action of lactate. The treatment with AOA also inhibited glucose-stimulated insulin secretion in INS-1 cells. This suggests that the glycerol phosphate shuttle is less active than the malate-aspartate shuttle in the regeneration of cytosolic NAD⁺ consumed by the glycolysis in INS-1 cells. In rat islets a 20-fold higher concentration of AOA inhibits glucose-stimulated insulin secretion by about 50% (32, 33), whereas this compound is less efficient in mouse islets (9). It appears, thus, that in mouse β cells the activity of the glycerol

phosphate shuttle can substitute for the malate-aspartate shuttle, whereas this is not the case in INS-1 cells, despite high activity of mGPDH (8, 34). The reason for this discrepancy may lie in substrate availability or in other regulatory factors.

In contrast to pyruvate, lactate did not elicit insulin secretion in islets infected with AdCAMCT-1 alone. The simultaneous overexpression of MCT-1 and LDH-A caused marked insulin secretion in response to lactate, which was associated with increased lactate oxidation by the islets. The combined results from INS-1 cells and islets demonstrate that endogenous LDH activity does not allow generation of sufficient amounts of pyruvate, the essential intermediate, to accomplish insulin secretion by lactate. This can be brought about by LDH-overexpression. In INS-1 cells, high concentrations of extracellular lactate elicit insulin secretion by forcing the LDH reaction to provide sufficient quantities of pyruvate even without LDH-A overexpression.

It has been reported that more than 90% of glucose-derived pyruvate enters into the mitochondria in the β cell (6). Low activities of LDH and MCT might contribute to the efficient channeling of glucose-derived pyruvate into the mitochondria by preventing pyruvate from escaping from the cell. However, neither glucose metabolism nor glucose-stimulated insulin secretion was affected in both INS-1 cells and rat

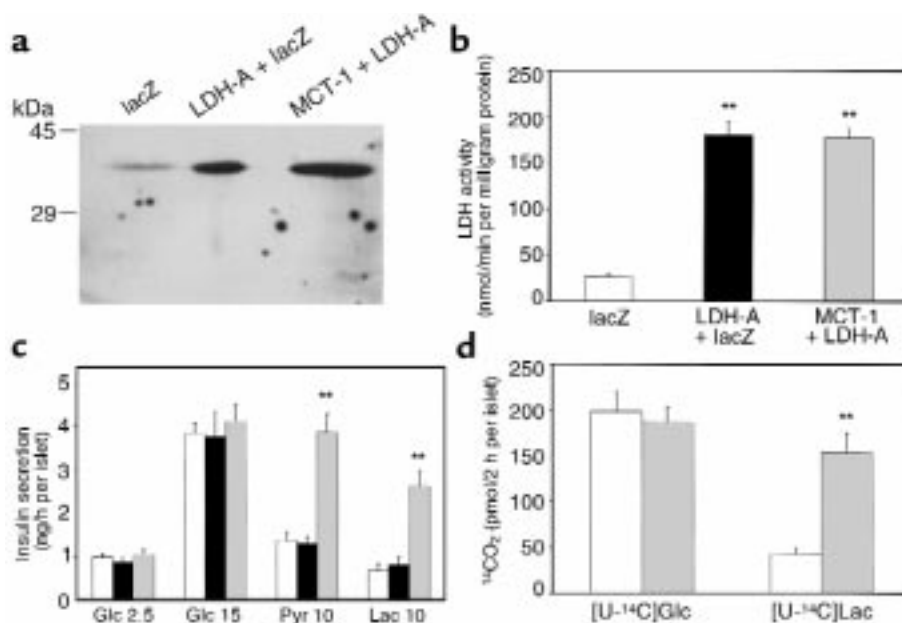


Figure 6

Co-overexpression of MCT-1 and LDH-A through recombinant adenoviruses and its effects on isolated islets. (a) One hundred twenty islets infected with either AdCALacZ, AdCALDH-A plus AdCALacZ (AdCALacZ was combined to adjust total virus amounts), or AdCAMCT-1 plus AdCALDH-A were used for immunoblot analyses. The result is representative of 2 independent experiments. (b) Lysates of 100 islets infected with recombinant adenoviruses were assayed for LDH activity. Data show the mean $\pm$ SEM of 3 independent experiments. **Difference from control islets at $P < 0.01$. (c) Ten islets infected with recombinant adenoviruses in each tube were incubated in KRBH with the indicated stimuli for 60 minutes. Data show the mean $\pm$ SEM of 4 independent experiments. Statistical significance between islets overexpressing both MCT-1 and LDH-A and control islets were obtained at 10 mM pyruvate and 10 mM lactate ($P < 0.01$). (d) ¹⁴CO₂ formation during 2-hour incubation with 15 mM [U-¹⁴C]glucose or 10 mM [U-¹⁴C]lactate were measured. Data show the mean $\pm$ SEM of 3 independent experiments. **Difference from control islets at $P < 0.01$. Open bars, islets infected with AdCALacZ; filled bars, islets infected with AdCALDH-A plus AdCALacZ; shaded bars, islets infected with AdCAMCT-1 plus AdCALDH-A.

islets after overexpression of LDH-A and/or MCT-1 (Figures 3 and 6c). Moreover, lactate output was not changed significantly in INS(LDH) cells exposed to 15 mM glucose after overexpression of LDH-A and MCT-1 (data not shown). These observations suggest that efficient metabolic channeling of glucose-derived pyruvate to the mitochondria is not dependent on low LDH and MCT activities. An attenuated glucose-stimulated insulin secretion was reported in LDH-A overexpressing stable clones of MIN6 cells relative to wild-type MIN6 cells, another well-differentiated tumoral β -cell line (35). A possible explanation for the differences between our results and the data obtained in MIN6 cells is that the stable overexpression of LDH-A might exert untoward effects rendering the cells less responsive to glucose. Note that glucose-stimulated insulin secretion was also unaffected in MIN6 cells transiently overexpressing LDH-A through a recombinant adenovirus (36). In any case, our results suggest that unknown mechanisms of efficient coupling of glycolysis to mitochondrial metabolism exist. Further studies are needed to reveal the mechanism of the efficient channeling of glycolytic intermediates to the mitochondria.

What would be the consequence if lactate would also act as a nutrient in β cells? Lactate is now considered to be an important energy source in several cells such as neurons and heart muscle cells, especially when circulating glucose is insufficient (37, 38). Plasma lactate levels in humans can increase up to nearly 10 mM during exercise (39). Such a high concentration of lactate could stimulate insulin secretion if the β cell would express high levels of MCT and LDH. The results here, therefore, suggest that low LDH and MCT activities protect the β cell from stimulatory effects of lactate, which could otherwise cause undesired insulin secretion during a catabolic state such as exercise. In other terms, low LDH and MCT activities confer specific selectivity to glucose of insulin release and avoid stimulation by pyruvate and lactate.

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Characterization of SotA and SotB, Two *Erwinia chrysanthemi* Proteins Which Modify Isopropyl- β -D-Thiogalactopyranoside and Lactose Induction of the *Escherichia coli lac* Promoter

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The expression, in *Escherichia coli*, of variants of the *Erwinia chrysanthemi* secretion genes *outB* and *outS* under the *P_{tac}* promoter is toxic to the cells. During attempts to clone *E. chrysanthemi* genes able to suppress this toxicity, I identified two genes, *sotA* and *sotB*, whose products are able to reduce the isopropyl- β -D-thiogalactopyranoside (IPTG) induction of the *E. coli lac* promoter. SotA and SotB belong to two different families of the major facilitator superfamily. SotA is a member of the sugar efflux transporter family, while SotB belongs to the multidrug efflux family. The results presented here suggest that SotA and SotB are sugar efflux pumps. SotA reduces the intracellular concentration of IPTG, lactose, and arabinose. SotB reduces the concentration of IPTG, lactose, and melibiose. Expression of *sotA* and *sotB* is not regulated by their substrates, but *sotA* is activated by the cyclic AMP receptor protein (CRP), while *sotB* is repressed by CRP. Lactose is weakly toxic for *E. chrysanthemi*. This toxicity is increased in a *sotB* mutant which cannot efficiently efflux lactose. This first evidence for a physiological role of sugar efflux proteins suggests that their function could be to reduce the intracellular concentration of toxic sugars or sugar metabolites.

Active efflux has been shown to be the cause of the drug resistance of many bacteria. Some of the proteins performing this efflux can deal with one type or a narrow range of structurally related compounds, like the *Escherichia coli* tetracycline exporter TetB (20). Other proteins are able to extrude from the cell a broad variety of unrelated compounds (antibiotics, quaternary amines, and basic dyes). The resistance conferred by these proteins is called multidrug resistance (MDR). The activity of these pumps gives bacterial pathogens a significant resistance to widely used antibiotics. The gram-negative MDR proteins belong to four different families of transport protein (23, 26). (i) The first is the major facilitator superfamily (MFS). This large group of proteins, which contains membrane transport proteins from bacteria to higher eukaryotes, is involved in the symport, antiport, or uniport of various substrates (25). It includes the well-studied MDR transporters QacA of *Staphylococcus aureus* and Bmr of *Bacillus subtilis*. These proteins contain 12 or 14 transmembrane segments. (ii) The second is the resistance nodulation division family (RND). Proteins of this family contain 12 transmembrane helices. The *E. coli* AcrB and the *Pseudomonas aeruginosa* MexB proteins belong to this group (19, 27). (iii) The third, the Smr family, exemplified by the *S. aureus* Smr protein, contains proteins smaller than those of the former groups, which possess four transmembrane segments (11). (iv) The fourth, the recently described MATE family, is exemplified by the *Vibrio parahaemolyticus* NorM protein (6).

The specificity of these MDR proteins is generally very broad, and a large spectrum of compounds can be effluxed (10). However, their substrates have been sought among antibiotics and drugs (basic dyes, sodium dodecyl sulfate [SDS], quaternary ammonium compounds, carbonyl cyanide *m*-chloro-

rophenylhydrazone, etc.). Recent experiments have shown that other types of molecules, such as sugars, can be pumped out of bacteria. The CmlA efflux pump, which can export a wide variety of antibiotics from different families, can efflux the galactoside isopropyl- β -D-thiogalactopyranoside (IPTG), a gratuitous inducer of genes controlled by the LacI repressor (3). Induction-based suppression of toxic effects has led to the discovery of two *E. coli* genes, belonging to two different families, encoding sugar efflux proteins. The first of these genes, *ydeA*, is a member of the MFS with 12 transmembrane segments (5, 7). Its expression prevents accumulation of arabinose and IPTG in the cytoplasm. The second one, *setA* (*yabM*), belongs to a new subfamily of the MFS, the SET (sugar efflux transporter) family (18), which includes two other *E. coli* proteins (YieO and YieK), a protein from *Yersinia pestis*, and one from *Deinococcus radiodurans*. SetA is able to prevent the accumulation of lactose and IPTG in *E. coli* cells. Thus, sugars and glycosides can both be substrates of efflux pumps.

During a search for *Erwinia chrysanthemi* genes whose products are able to suppress the toxic effect due to the expression of the *E. chrysanthemi outS* and *outB* genes cloned under the *P_{tac}* promoter, I identified two genes able to reduce the intracellular IPTG concentration of the bacteria. This leads to a reduction in the toxicity resulting from the accumulation of OutS and OutB in the bacteria. I also showed that their products are able to prevent the accumulation of several sugars in the bacteria.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and drug resistance assays. The strains and plasmids used in this study are listed in Table 1. *E. chrysanthemi* and *E. coli* strains were grown at 30 and 37°C, respectively, unless otherwise stated. The media used were Luria broth (LB) or M63 minimal medium supplemented with a carbon source (0.2%), and for cloning experiments, the following antibiotics were used at the concentrations indicated: ampicillin, 100 μ g/ml; kanamycin, 50 μ g/ml; chloramphenicol, 20 μ g/ml; and tetracycline, 10 μ g/ml. The resistance of *E. coli* strains containing cloned genes to drugs was tested by spotting 10 μ l of a 10⁵-fold dilution of an overnight culture on LB plus ampicillin plates containing

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>E. chrysanthemi</i>		
3937	Wild type	Laboratory collection
A350	<i>lmrT(Con) lacZ</i>	15
A2507	<i>lmrT(Con) lacZ crp::Cm</i>	29
A3145	<i>lmrT(Con) lacZ sotA::uidA-kan</i>	This work
A3501	<i>lmrT(Con) lacZ sotB::uidA-kan</i>	This work
<i>E. coli</i>		
NM522	<i>supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5 r_K⁻ m_K⁻ F' proAB lacI^a ΔlacZM15</i>	Stratagene
P4X	Hfr <i>metB relA1 spoT1</i>	Laboratory collection
NA1	F ⁻ <i>araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	14
	<i>uxaB::Mud(Ap^r lac)</i>	
1701	F ⁻ <i>araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	J. Beckwith
	<i>(rbs-lac)op6.16</i>	
9665	F ⁻ <i>araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	1
	<i>trp::(malK-lac)1</i>	
Plasmids		
pACT3	Cm ^r <i>Ptac</i> ori p15A	9
pEXT20	Ap ^r <i>Ptac</i>	9
pACTPB	pACT3 expressing <i>pelB</i>	This work
pABSC3	pACT3 expressing <i>pelB^{sp}-outB</i>	G. Condemine and V. E. Shevchik
pABCT2	pACT3 expressing <i>outB</i>	G. Condemine and V. E. Shevchik
pACTS1	pACT3 expressing <i>pelB^{sp}-outS</i>	32
pBAD18	Ap ^r P _{BAD}	12
pBADuid	<i>uidA</i> -kan cloned in the pBAD18 polylinker	This work
pBR322	Tet ^r Ap ^r	4
pBRS1	pBR322 expressing <i>sotA</i>	This work
pBRS6	pBR322 expressing <i>sotB</i>	This work
pSU9	Cm ^r	2
pS1A	pSU9 expressing <i>sotA</i>	This work
pS6E	pSU9 expressing <i>sotB</i>	This work
pUC18	Ap ^r	Biolabs
pUCS1	pUC18 expressing <i>sotA</i>	This work
pUCS6	pUC18 expressing <i>sotB</i>	This work
pULB110	Tet ^r Ap ^r (Mu 3A)	34

various dilutions of the tested compound. The ability of the strain to form single colonies was recorded after 24 h.

Genetic techniques. Transduction with phage ϕ EC2 was performed as described by Résibois et al. (28). Genetic mapping was performed with pULB110 as described by van Gijsegem and Toussaint (34). Marker exchange recombinations were obtained after growth in a low-phosphate medium as described by Roeder and Collmer (30).

Enzyme assays. β -Glucuronidase assays were performed with toluenized cells grown to the exponential phase with *p*-nitrophenol- β -D-glucuronate as the substrate (24). β -Galactosidase assays were performed with toluenized cells grown to the exponential phase with *o*-nitrophenol- β -D-galactose as the substrate (21).

Recombinant DNA techniques. Preparations of chromosome and plasmid DNA, restriction digestions, ligations, DNA electrophoresis, transformations, and electroporations were carried out as described by Sambrook et al. (31). Sequencing was performed by Genome Express SA (Grenoble, France).

SDS-PAGE and Western blotting. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (17). After electrophoresis, proteins were electroblotted onto nitrocellulose in a semidry apparatus at 1 mA/cm² for 1 h in transfer buffer containing 40 mM glycine, 50 mM Tris, 0.4% SDS, and 10% methanol. The nitrocellulose was then saturated and incubated with antibodies and developed with the ECL enhanced chemiluminescence detection kit (Pharmacia-Amersham Biotech).

Nucleotide sequence accession number. The complete DNA sequences of *sotA* and *sotB* have been deposited in the EMBL database under accession no. AJ249180 and AJ249181, respectively.

RESULTS

Isolation of two genes suppressing the toxicity of PelB^{sp}-OutS and PelB^{sp}-OutB. OutS and OutB are two components of the *E. chrysanthemi* Out secretion machinery, involved in the

secretion of pectinases in the external medium (8). OutS, an outer membrane lipoprotein, is a chaperone of the secretin OutD (32, 33). OutB is an inner membrane protein that interacts with OutD (G. Condemine and V. E. Shevchik, unpublished data). We have constructed variants of *outS* and *outB* in which the region coding for their N-terminal anchors has been replaced by the region coding for the PelB signal sequence (32; G. Condemine and V. E. Shevchik, unpublished data). These constructs have been cloned under the *Ptac* promoter of plasmid pACT3. When *E. coli* cells containing these plasmids were grown in the absence of IPTG at 30°C, the resulting proteins (PelB^{sp}-OutS and PelB^{sp}-OutB) were correctly synthesized, processed, and accumulated in the periplasm. When grown at 37°C in the presence of IPTG, the cells were unable to form colonies on plates and were lysed in liquid medium. To identify proteins interacting with OutS and OutB, I tried to isolate *E. chrysanthemi* genes which, expressed on a multicopy plasmid, could suppress the toxic effect due to the overexpression of PelB^{sp}-OutS and PelB^{sp}-OutB. An *E. chrysanthemi* gene library constructed in plasmid pUC18 was introduced into *E. coli* strain NM522 containing plasmid pACTS1 (*pelB^{sp}-outS*). A total of 6×10^4 transformants were spread onto LB-ampicillin-chloramphenicol-IPTG plates that were incubated overnight at 37°C. Six transformants were able to grow on this medium. Restriction mapping of the six plasmids suppressing the toxicity of PelB^{sp}-OutS showed that they fall into two

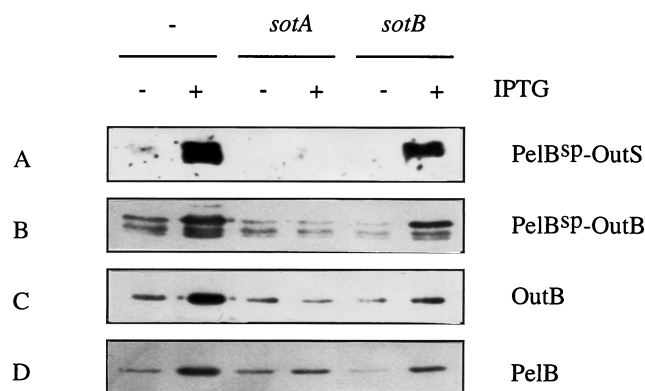


FIG. 1. Effect of the presence of *sotA* and *sotB* on the expression of genes cloned into plasmid pACT3. *E. coli* NM522 cells containing plasmids pACTS1 (*pelB^{SP}-outS*) (A), pABSC3 (*pelB^{SP}-outB*) (B), pABTC2 (*outB*) (C), and pACTPB (*pelB*) (D) were transformed with plasmids pUC18, pUCS1 (*sotA*), and pUCS6 (*sotB*). Cells were grown for 3 h in the absence or presence of 10^{-4} M IPTG. Aliquots were loaded onto SDS-PAGE gel and immunoblotted with anti-OutS (A), anti-OutB (B and C), and anti-PelB (D) antibodies.

groups. Five contained a common region, and one had a totally different restriction map. One plasmid of each type was retained for further studies. The two genes suppressing toxicity were named *sotA* and *sotB* (for suppressor of Out toxicity). The plasmids bearing *sotA* and *sotB* were introduced into NM522 containing plasmid pABSC3 (*pelB^{SP}-outB*). Both transformants were able to grow at 37°C in the presence of IPTG, indicating that the *sotA* and *sotB* genes are also able to suppress the toxicity induced by PelB^{SP}-OutB.

***sotA* and *sotB* modify IPTG induction of the *Ptac* promoter.** To determine the mode of action of SotA and SotB, I estimated the amount of PelB^{SP}-OutS and PelB^{SP}-OutB in cells expressing, or not, SotA and SotB in the absence or presence of IPTG. While in the absence of IPTG no significant variation was observed, the amounts of both PelB^{SP}-OutS and PelB^{SP}-OutB were reduced with either SotA or SotB. This effect was more pronounced when SotA was present (Fig. 1). To test whether the effect of *sotA* and *sotB* was specific to PelB^{SP}-OutS and PelB^{SP}-OutB, I quantified the products of two other genes (*outB* and *pelB*) cloned under the *Ptac* promoter of plasmid pACT3 and expressed under the same conditions. Overexpression of wild-type OutB or PelB from pACT3 is not toxic for the bacteria. The amount of OutB or PelB was also reduced when produced with SotA or SotB (Fig. 1). This reduction could result from a decrease in the copy number of plasmid pACT3, from a reduced induction of the *Ptac* promoter, or from an increased degradation of mRNAs or proteins. I first checked that the copy number of pACT3 was unchanged in the presence of *sotA* or *sotB* (data not shown). To test the other hypotheses, we expressed OutB under its own promoter in plasmid pBR322 and under the *tac* promoter of plasmid pEXT20. pEXT20 is a pBR322 derivative that contains the expression cassette comprising the *Ptac* promoter of plasmid pACT3. While expression of OutB from pBR322 was not modified by the presence of SotA and SotB, even in the presence of IPTG, expression from pEXT20 was reduced (data not shown). This result indicates that SotA and SotB do not modify the stability of RNAs or proteins, but they do influence the IPTG induction of the *Ptac* promoter of pEXT20 or pACT3. Induction of *pelB* cloned into pACT3 was tested by using increasing amounts of IPTG. Induction of PelB synthesis was observable with concentrations of IPTG as low as 10^{-6} M in the control strain. A 10^{-5} M concentration of IPTG was required to induce PelB synthesis when *sotB* was present, and

10^{-4} M IPTG was required when *sotA* was present (Fig. 2). Thus, SotA and SotB could decrease the sensitivity of *Ptac* to IPTG or reduce the intracellular concentration of IPTG.

The products of *sotA* and *sotB* are putative membrane transport proteins. The DNA fragment containing the *sotA* gene, reduced to 1.6 kb by subcloning, was sequenced. It contains one putative open reading frame of 1,179 nucleotides, encoding a protein of 42,433 Da with 12 putative transmembrane domains. A similarity search in data banks revealed that it belongs to the recently identified sugar efflux transporter family, a subgroup of the MFS of membrane transporters. A strong homology exists with the proteins SetB (YeiO) (68% identity), YicK (61% identity), and SetA (YabM) (53% identity) of *E. coli*. SetA and SetB can efflux lactose and/or IPTG from *E. coli* cells.

The *sotB* gene has been localized by subcloning on a 2.1-kb DNA fragment which has been sequenced. It contains one putative complete open reading frame of 1,185 nucleotides, encoding a protein of 41,960 Da with 12 putative transmembrane domains. SotB belongs to another subfamily of the MFS which contains MDR proteins. SotB presents homology with the chloramphenicol resistance protein of *Streptomyces lividans* (CmlR) which exports the drug, the AraJ protein of *E. coli*, and the YdeA proteins of *E. coli* and *Haemophilus influenzae*. The highest identity was observed with YdeA of *E. coli* (61%). The *E. coli* YdeA protein is able to export arabinose and IPTG from the bacteria.

SotA and SotB can export several sugars. The reduced induction of IPTG of the *Ptac* promoter in the presence of SotA and SotB and the homology of these proteins with sugar exporters suggested that SotA and SotB are sugar efflux pumps. To test this hypothesis and to determine the substrates of these pumps, we measured whether the presence of SotA and SotB on a plasmid in *E. coli* modifies the induction of sugar utilization operons by their substrate. Plasmids bearing *sotA* and *sotB* were introduced into *E. coli* P4X, and *lacZ* induction was monitored. A reduction in the induction level of the *lacZ* gene by IPTG was observed in the presence of SotA or SotB (Fig. 3A and B). However, the concentration at which this effect was observable was 10-fold lower with SotB than with SotA (3×10^{-6} M versus 4×10^{-5} M). The *lac* operon is also inducible by lactose and melibiose (21). While induction of *lacZ* by these compounds was barely modified in the presence of SotA, it was clearly diminished by SotB (Fig. 3C, D, and E). Efflux of galacturonate, ribose, and maltose was tested by measuring the induction of an *uxaB-lacZ* fusion, an *rbs-lacZ* fusion, and a *malK-lacZ* fusion in the presence of *sotA* and *sotB*. No modi-

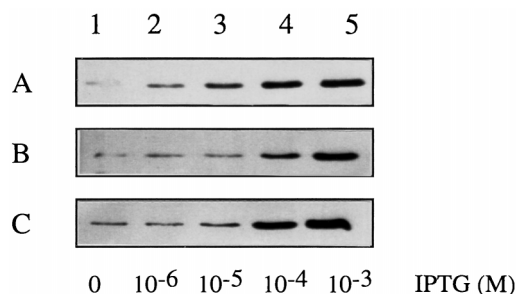


FIG. 2. Effect of the presence of *sotA* and *sotB* on the IPTG induction of *pelB* cloned into pACT3. *E. coli* NM522 cells containing pACTPB (*pelB*) and pUC18 (A), pUCS1 (*sotA*) (B), or pUCS6 (*sotB*) (C) were grown in LB medium with 0 (lane 1), 10^{-6} (lane 2), 10^{-5} (lane 3), 10^{-4} (lane 4), or 10^{-3} M IPTG (lane 5). Aliquots were loaded onto an SDS-PAGE gel (10% polyacrylamide) and immunoblotted with anti-PelB antibody.

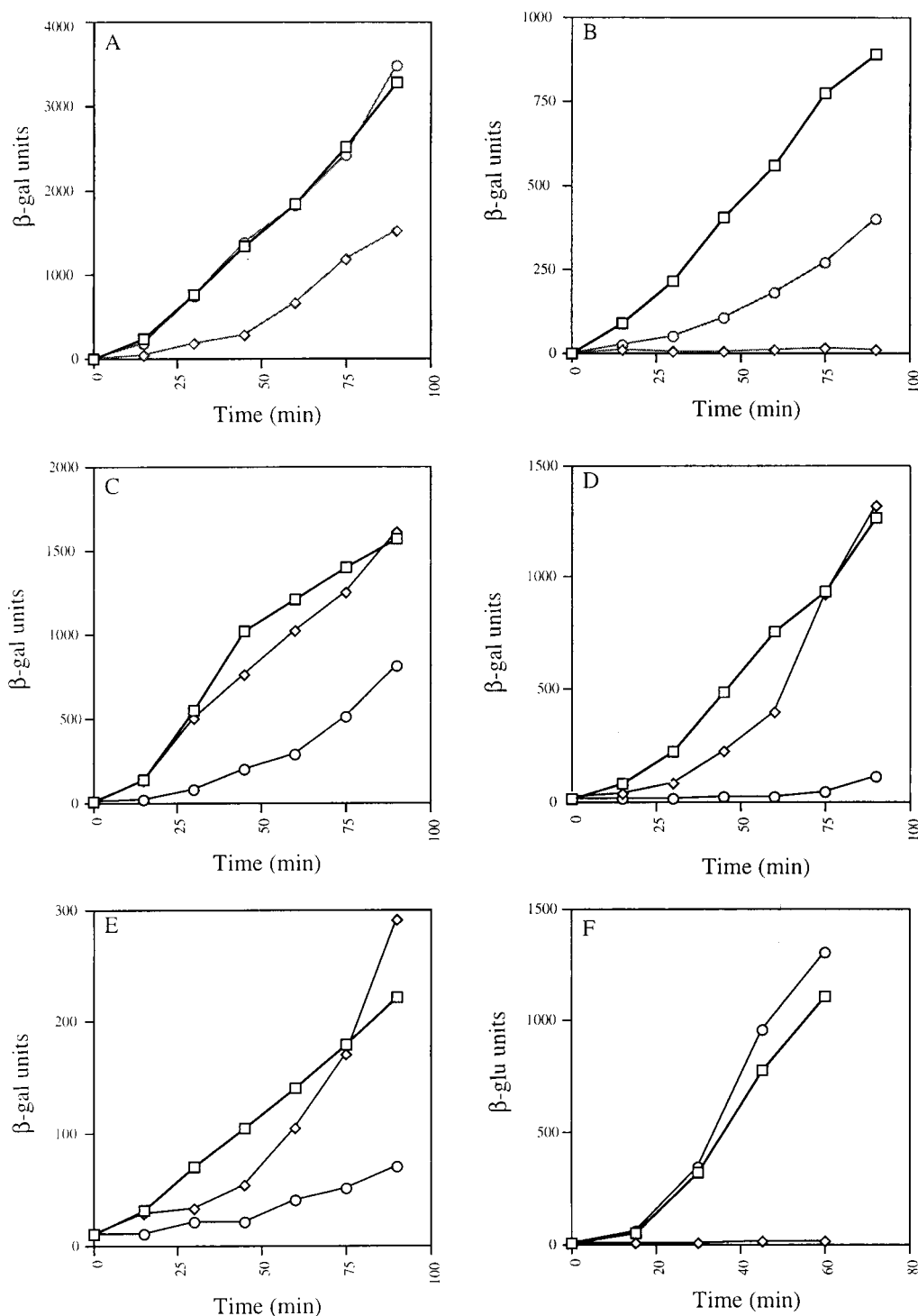


FIG. 3. Effect of the presence of *sotA* and *sotB* on the induction of the *lac* and *ara* promoter in *E. coli* by various compounds. Strains P4X (induction by IPTG, lactose, or melibiose) containing plasmid pBR322 (□), pBRS1 (*sotA*) (◇), or pBRS6 (*sotB*) (○), or NM522/pBADuid (induction by arabinose) carrying plasmid pSU9 (□), pS1A (*sotA*) (◇), or pS6E (*sotB*) (○) were grown in LB medium to an optical density at 600 nm of 0.5. Inducer was added at the concentration indicated, and the β-galactosidase (β-gal) or β-glucuronidase (β-glu) activity of the culture was measured over time. (A) IPTG (4×10^{-5} M). (B) IPTG (3×10^{-6} M). (C) Lactose (20 mg/liter). (D) Lactose (5 mg/liter). (E) Melibiose (40 mg/liter). (F) Arabinose (4 mg/liter).

fication of the induction rate of these fusions was observed, indicating that these sugars are not substrates of SotA or SotB. Efflux of arabinose was tested by measuring the induction of β-glucuronidase encoded by the *uidA* gene, which was cloned under the *Para* promoter of plasmid pBAD18. The presence of

SotA, but not of SotB, was able to prevent, very efficiently, induction by arabinose of *uidA* expression (Fig. 3F).

The multidrug transporter CmlA is able to confer resistance to a wide range of drugs and also to efflux IPTG (3). To test whether SotA and SotB could also efflux drugs, I compared the

TABLE 2. Expression of *sotA-uidA* and *sotB-uidA* fusions in the presence of various sugars

Growth condition ^a	Additional mutation	β-Glucuronidase activity ^b	
		<i>sotA-uidA</i>	<i>sotB-uidA</i>
Glycerol	— ^c	141 ± 5	16 ± 2
Glycerol + lactose	—	135 ± 7	18 ± 2
Glycerol + IPTG	—	141 ± 14	12 ± 2
Glycerol + melibiose	—	140 ± 9	49 ± 14
Glycerol + arabinose	—	104 ± 8	38 ± 12
Glycerol + galacturonate	—	216 ± 8	48 ± 6
Glycerol + rhamnose	—	147 ± 9	15 ± 2
Glycerol + maltose	—	152 ± 6	13 ± 1
Glucose	—	99 ± 7	62 ± 6
Glucose	<i>crp</i>	72 ± 6	125 ± 6

^a Bacteria were grown in M63 medium with 0.2% glycerol or glucose as the carbon source and 0.2% of the sugars tested or 1 mM IPTG.

^b β-Glucuronidase activity is expressed in nanomoles of *p*-nitrophenol formed per minute per milligram (dry weight) of bacteria. Each value is the average of four determinations.

^c —, none.

resistance of strain NM522, with or without *sotA* and *sotB*, to 19 antibiotics and drugs (tetracycline, chloramphenicol, rifampin, nalidixic acid, novobiocin, kanamycin, spectinomycin, erythromycin, fusidic acid, trimethoprim, gentamicin, vancomycin, kasugamycin, crystal violet, SDS, ethidium bromide, oleic acid, coumaric acid, and ferric acid). The presence of *SotA* and *SotB* did not change the MICs of these compounds. Thus, *SotA* and *SotB* seem to efflux specifically sugars.

Analysis of *sotA* and *sotB* transcription. *sotA* and *sotB* mutants were constructed by reverse genetics. A *uidA*-kan cassette was inserted into the unique *Sma*I site of *sotA* and into the unique *Bam*HI site of *sotB*. Constructs in which the *uidA* gene was in the same orientation as *sotA* or *sotB* were retained, introduced into *E. chrysanthemi*, and recombined into the chromosome. The operon fusions obtained were used to study *sotA* and *sotB* transcription. Synthesis of multidrug transporters is often induced by one of the drugs they expel (22). Thus, we measured the expression of the *sotA-uidA* and *sotB-uidA* fusions in the presence of various sugars (Table 2). Expression of *sotA* was not induced by its substrates, IPTG and arabinose. Among the other sugars tested, only galacturonate gave a 1.5-fold induction. The presence of a *crp* mutation reduced by twofold the expression of *sotA*. The expression of *sotB* was not induced by lactose or IPTG, but increased fourfold in the presence of glucose. It was also induced to a lesser level by other metabolizable sugars, such as galacturonate, arabinose, and melibiose (Table 2). The fusion was expressed at an eightfold-higher level in a *crp* mutant. This suggested a repression of *sotB* by cyclic AMP receptor protein (CRP). A CRP binding site, which contains 9 out of the 10 nucleotides of the consensus (TGTGAN₆TCGCA), can be found at position 533 to 548, 65 bases upstream of the *sotB* initiation codon.

Analysis of *E. chrysanthemi sotA* and *sotB* mutants. The presence of lactose in the growth medium was detrimental to the growth of *E. chrysanthemi* A350, the strain used in this study (Fig. 4). It has been designed for the construction of *lacZ* gene fusion (15). Lactose uptake has been increased by the derepression of the expression of the broad-substrate-specificity transporter *lmrT* (involved in lactose, melibiose, and raffinose entry), and the *lacZ* gene has been inactivated. Thus, this strain is unable to metabolize lactose. The inhibitory effect of lactose on the growth of the *sotA* and *sotB* mutant was compared with that observed with A350. While the growth of the *sotA* mutant was equivalent to that of A350, the *sotB* mutant

showed reduced growth, indicating that the presence of an efficient lactose efflux pump, *SotB*, is sufficient to reduce, at least partially, the lactose toxicity toward *E. chrysanthemi*.

sotA and *sotB* have been mapped on the *E. chrysanthemi* chromosome. Chromosomal mobilization mediated by the plasmid pULB110 was used for conjugation with various polyauxotrophic recipient strains. Both genes were localized between the *his-1* and *gal-1* markers (16). *sotA* and *sotB* are cotransducible by phage φEC2 with a frequency of 63%, indicating that they are probably located within 20 kb of each other.

DISCUSSION

The multicopy suppression strategy I used, which involved looking for genes whose products could suppress the toxicity of PelB^{SP}-OutS or PelB^{SP}-OutB, was aimed at finding proteins that interact with OutS and OutB. This approach led to the identification of two genes that are not related to the Out secretion system, but whose products are able to reduce the intracellular concentration of the *lac* gene inducer, IPTG. The presence of *sotA* or *sotB* reduces the expression of any gene cloned under the *P_{tac}* promoter of plasmid pACT3 by lowering the intracellular concentration of IPTG. The results presented here suggest that *SotA* and *SotB* are sugar efflux proteins. The other known sugar efflux proteins have been isolated fortuitously by similar cloning strategies: *cmlA* and *setA* reduce induction by IPTG of toxic genes cloned under the *P_{tac}* promoter (3, 18), and *ydeA* prevents the arabinose-dependent expression of a toxic protein expressed under the control of the arabinose *P_{BAD}* promoter (5, 7). Thus, the use of a multicopy suppression strategy when the toxic protein synthesis is under the control of an inducible promoter often leads to the identification of genes able to expel the inducer. New sugar efflux transporters could be identified, by the same strategy, by cloning toxic genes in other sugar-inducible promoter-based expression vectors.

Up to now, no physiological function has been assigned to

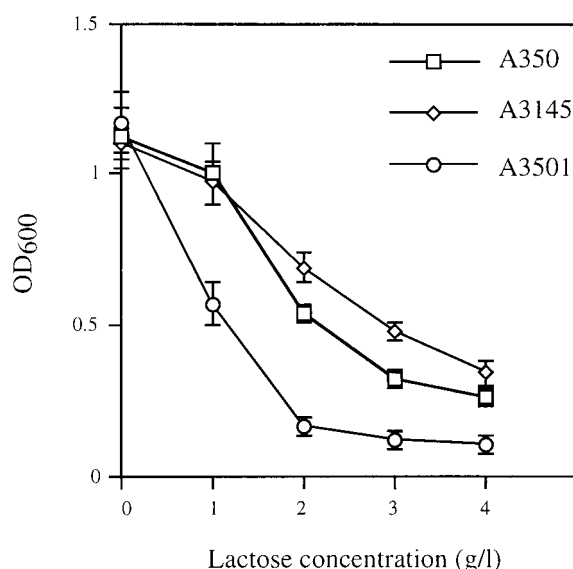


FIG. 4. Growth of strain A350 and of *sotA* and *sotB* mutants in the presence of lactose. M63 medium containing 0.2% glycerol and various concentrations of lactose was inoculated at a 1:100 dilution with a culture of strain A350, A3156, or A3501. After 16 h of growth, the optical density at 600 nm (OD_{600}) was measured.

sugar efflux pumps. The results presented here show that one of their functions could be to remove toxic sugars from the cytoplasm. Although the cause of lactose toxicity in *E. chrysanthemi* is not known, it is clear that the absence of an efflux system able to reduce the intracellular concentration of this sugar has a detrimental effect on the growth of the bacteria. Accumulation of nonmetabolizable sugars or of sugar metabolites, such as sugar phosphates, can have growth-inhibitory effects. The sugar efflux transporters could participate in the regulation of the intracellular concentration of these toxic compounds. The presence of three SET members in *E. coli* could indicate that this strategy of detoxification is widely used by bacteria. Huber et al. (13) have shown that when *E. coli* cells are grown in the presence of lactose, a large proportion of the β -galactosidase products are found in the culture medium. Sugar efflux pumps could help maintain an intracellular concentration of sugar within a range compatible with the biochemical and regulatory metabolism of the bacteria. Recently, amino acid efflux pumps have been described (35). It would be interesting to investigate whether the intracellular concentrations of other compounds could also be regulated by efflux pumps.

Initially, SotA and SotB may seem redundant, but the specificity of the two transporters appears different. IPTG, melibiose (6-O- α -D-galactopyranosyl-D-glucose), and lactose [β -D-galactose (1-4)- α -glucose] are apparently good substrates for SotB. Thus, SotB can be considered as an exporter of β -galactosides. The specificity of SotA seems to be wider, since it can apparently efflux IPTG, lactose, and arabinose, a pentose. The differences in substrate specificity may contribute as well to differences in the respective roles of SotA and SotB. Screening of other sugar efflux activities by SotA and SotB will show if they have a narrow specificity, recognizing only one type of glycoside, or if they are multisugar efflux pumps, with a specificity as broad as that of some multidrug efflux pumps. The absence of a specific regulation of *sotA* and *sotB* expression by lactose or arabinose and their control by the global regulator CRP, the homology of SotB with MDR proteins, suggest that they could have a broad specificity, contributing to the efflux of many sugars from the cytoplasm. However, in contrast to CmlA, they do not seem to be able to export drugs and antibiotics.

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