

Synthesis of Pyruvate Dehydrogenase in *Staphylococcus aureus* Is Stimulated by Osmotic Stress

Oddur Vilhelmsson† and Karen J. Miller*

Department of Food Science, The Pennsylvania State University, University Park, Pennsylvania, 16802

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The pyruvate dehydrogenase multienzyme complex (PDHC) was found to be upregulated by osmotic stress in the osmotolerant pathogen *Staphylococcus aureus*. Upregulation was detectable in the levels of both activity and protein and was judged to be about fourfold when sodium chloride was used to adjust the water activity (a_w) of the growth medium to 0.94. The upregulation of the PDHC was also found to be humectant dependent and was greatest when impermeant, nonmetabolizable humectants were used to adjust a_w . Further experiments provided evidence that in addition to osmotic upregulation, the PDHC complex is also subject to catabolite repression, thus providing a possible explanation for the observation that high concentrations of carbohydrates are generally more inhibitory to the growth of this bacterial pathogen than are high concentrations of salts.

Staphylococcus aureus, an important human pathogen, is considered a highly osmotolerant bacterium and is able to grow at a water activity (a_w) of as low as 0.86 (33). Its osmotolerance may be an important determinant of its ability to grow in foods and on human skin, from where it can spread and/or form toxins and cause disease. Although osmoadaptation of *S. aureus* has been studied fairly extensively in the past (8, 9, 29, 30, 38, 42, 43, 46), it remains unclear why this organism is considerably more osmotolerant than other bacterial pathogens. In fact, thus far, studies have revealed an osmoadaptive process apparently typical for bacteria in general: namely, the accumulation of glycine betaine, proline, or other common compatible solutes through transport from the growth medium (25, 29, 39) and increased synthesis of stress proteins such as chaperones (31) and alkyl hydroperoxide reductase C (8). However, recent studies have given indications that the regulation of stress gene expression may be somewhat unusual in the staphylococci. For example, expression of the alternative sigma factor σ^B is repressed during osmotic stress in *S. aureus* (13) while it is strongly induced in *Bacillus subtilis* under those conditions (11, 45). Although a few studies have attempted to identify osmotically upregulated proteins in *S. aureus* (8, 31, 44), this field still remains relatively unexplored.

A major goal of the present study was to investigate changes in protein composition of this osmotolerant organism in response to osmotic stress and to identify proteins important for its osmoadaptation. Herein, we present evidence that the pyruvate dehydrogenase multienzyme complex (PDHC) of *S. aureus*, an enzyme of central importance in this organism's primary metabolism, is upregulated by osmotic stress. The implications of this finding with respect to the osmoadaptation of *S. aureus* and the regulation of its metabolic activity are discussed.

MATERIALS AND METHODS

Strains and media. *S. aureus* strain ATCC 12600 was obtained from the American Type Culture Collection (Manassas, Va.) and grown in Trypticase soy broth (TSB; Difco Laboratories, Detroit, Mich.) at 37°C. The a_w values of media were measured in duplicate at room temperature in an Aqua Lab CX-2 water activity meter (Decagon, Pullman, Wash.). For testing growth on different carbon sources and measuring glucose consumption, the defined medium (DFM) described previously (44a) was used, except proline levels were set at 5 mM and glucose was replaced, where appropriate, with other potential carbon sources (D-fructose, D-arabinose, L-arabinose, D-sucrose, D-mannose, D-xylose, glycine betaine, glycerol) at 0.5% (wt/vol).

Isolation and two-dimensional (2D) electrophoresis of membrane proteins. Since osmoadaptation is a response to changing environmental conditions, it was decided to focus on the part of the cellular protein profile most exposed to the environment, namely plasma membrane-associated proteins. Cells were grown in approximately 1 liter of TSB or TSB adjusted to an a_w of 0.95, 0.92, or 0.89 with glycerol, NaCl, or sucrose; cells were harvested at the late exponential or stationary growth phase. Harvesting (by centrifugation), lysis (by osmotic disruption of protoplasts), and isolation of plasma membranes (by centrifugation) were performed essentially as described by Sprott et al. (36), except lysostaphin (1.0 U ml of culture⁻¹) was used instead of lysozyme and sorbitol (50% [wt/vol]) was used instead of sucrose to control osmotic pressure. Membrane proteins were solubilized according to the method of Ames and Nikaido (7). An aliquot containing approximately 200 μ g of membrane protein, as determined by a modified Lowry method (23), was diluted with 2 volumes of an isoelectrofocusing buffer described by Ames and Nikaido (7) containing 3% (vol/vol) IGEPAL CA-630 (Sigma Chemical Company, St. Louis, Mo.) instead of NP-40. The samples were electrophoresed in the first dimension on 4% polyacrylamide (PA) tube gels (1-mm diameter) for 16,500 V · h. The second dimension was done on a 4% PA stacking gel and a 10% PA running gel at 35 mA per gel. The gels were stained with 0.2% Coomassie brilliant blue R-250 in 5:4:1 distilled water:methanol:acetic acid and destained in 0.5 M NaCl (37), except when the gels were to be used for sequencing, in which case the gels were stained with 0.05% Coomassie brilliant blue R-250 in 20% methanol (sequencing grade) and 0.5% acetic acid. Destaining was performed in 30% sequencing grade methanol, and the gels were stored in distilled water. The gels were scanned with a Bio-Rad (Hercules, Calif.) model GS-670 imaging densitometer and analyzed with a Molecular Analyst 2DFull software package (Bio-Rad).

Spot elution and N-terminal sequencing. Selected spots were excised from four 2D gels, homogenized in a 1.5-ml-capacity tissue grinder, and extracted by passive diffusion as described by Hames (18). The extracts were electrophoresed on a 4% PA stacking gel and a 10% PA running gel with 7 μ l of thioglycolic acid liter⁻¹ in the upper running buffer. The samples were then electroblotted onto a sequencing grade polyvinylidene difluoride membrane (Bio-Rad) according to protocols supplied by the manufacturer. N-terminal sequencing by Edman degradation was performed at the Macromolecular Core Facility at the Hershey Medical Center, Hershey, Pa.

* Corresponding author. Mailing address: 101 Borland Laboratory, Department of Food Science, The Pennsylvania State University, University Park, PA 16802. Phone: (814) 863-2954. Fax: (814) 863-6132. E-mail: kjm3@psu.edu.

† Present address: Department of Microbiology and Molecular Genetics, University of Texas—Houston Medical School, Houston, TX 77030.

Preparation of cell extracts and PDHC activity assays. Cultures (100 ml) were grown in TSB or TSB adjusted to a set a_w value (usually 0.94 or 0.96) with a humectant (D-sucrose, D-glucose, D-fructose, D-xylose, D-arabinose, L-arabinose, D-mannose, glycine betaine, sodium chloride, potassium chloride, sodium sulfate, glycerol, or polyethylene glycol with an average molecular weight of 8,000 [PEG 8000]). Cells were harvested by centrifugation ($10,000 \times g$ for 10 min), washed with 10 ml of a 100 mM sodium phosphate buffer (pH 6.6), and lysed by treatment with lysostaphin (100 U ml^{-1}) at 37°C for 60 min. After centrifugation ($10,000 \times g$ for 10 min), the pellet was resuspended in $150 \mu\text{l}$ of 100 mM sodium phosphate buffer (pH 6.6) containing 0.1% Triton X-100, 2 U of DNase, and 2 U of RNase. Protease inhibitor cocktail (catalogue no. P 8465; Sigma) was added according to the manufacturer's instructions, and the resulting suspension was incubated for 20 min at 37°C . The remaining cell debris was removed by centrifugation ($10,000 \times g$ for 10 min). Protein concentration in the extract was determined by a modified Lowry method (23) suitable for membrane protein preparations.

PDHC activity was assayed according to a procedure based on that of Seals and Jarett (34). Briefly, an aliquot containing a fixed amount (usually $100 \mu\text{g}$) of protein was added to a 20-ml-capacity serum vial that had been equipped with an elevated CO_2 -trapping cup holding a piece of Whatman no. 1 filter paper. To the sample, $155 \mu\text{l}$ of $5\times$ preincubation buffer (100 μM EDTA, 10 mM dithiothreitol, 5 mM ADP, 1% Triton X-100, 250 mM HEPES; pH 7.7) was added, and the resulting mixture was diluted to $775 \mu\text{l}$ with water and incubated at 37°C for 10 min. After incubation, $125 \mu\text{l}$ of assay buffer (10 mM NAD [Sigma], 2 mM coenzyme A [ICN Biomedicals, Aurora, Ohio], 2.5 mM thiamine pyrophosphate, 5 mM dithiothreitol, 5 mM MgCl_2 , 0.5% Triton X-100, 100 mM Tris-HCl; pH 8.0) was added, and the vials were sealed and incubated at 37°C for a further 5 min. After the second incubation, $100 \mu\text{l}$ of 1 mM (2 Mcpm ml^{-1}) [^{14}C]pyruvate (Amersham-Pharmacia, Piscataway, N.J.) (27 mCi mmol^{-1}) was injected into the solution and the vials were incubated at 30°C for 10 min, after which time the reaction was terminated by placing the vials on ice and adding 1 ml of ice-cold 3 M sulfuric acid to the solution. Finally, $150 \mu\text{l}$ of 1 M NaOH was injected onto the filters, and CO_2 was collected at room temperature with gentle shaking for 3 h. The filters were suspended in 10 ml of Ecocint H scintillation fluid (National Diagnostics, Atlanta, Ga.) and counted for 10 min in a Beckman (Palo Alto, Calif.) LS1701 scintillation counter. To eliminate the possibility that the measured activity resulted from the action of pyruvate-metabolizing enzymes other than the PDHC (e.g., pyruvate decarboxylase) and/or of pyruvate dehydrogenase alone (i.e., in isolation from the other PDHC components), assays were also performed in the absence of NAD, an essential cofactor for the PDHC whose turnover is mediated by the dehydrolipoamide dehydrogenase component of the PDHC (10).

Preparation of genomic DNA. Cells grown to late exponential growth phase in 6 ml of TSB were harvested by centrifugation at $10,000 \times g$ for 2 min. The cell pellet was resuspended in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 U of lysostaphin μl^{-1} , 1 U of RNase A μl^{-1} , 100 ng of proteinase K μl^{-1} , and 0.5% sodium dodecyl sulfate and incubated for 1 h at 37°C . Genomic DNA was extracted essentially as described by Moore (26).

PCR and sequencing. Preliminary sequence data were obtained from The Institute for Genomic Research (TIGR) website (<http://www.tigr.org>) and used to design oligonucleotide primers for amplification of a 1,129-bp fragment containing the *S. aureus pdhB* gene. Primers were synthesized by Integrated DNA Technologies (Coralville, Iowa) and were as follows: left primer, 5'-TGCCTCAAACTTAGCAGAACA-3' (binds the sequence 59 to 38 bases upstream of the *pdhB* initiation codon); right primer, 5'-CACGTTTTTGCCTCCTAAG-3' (binds the sequence 74 to 93 bases downstream of the *pdhB* termination codon). PCR was carried out in a GeneAmp 9600 thermocycler (Perkin-Elmer, Norwalk, Conn.). Initial denaturation was at 96°C for 5 min and was followed by 30 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 2 min, and extension at 72°C for 1 min, which in turn was followed by final extension at 72°C for 15 min and indefinite holding at 4°C . *Taq* polymerase (Promega, Madison, Wis.) was added after initial denaturation. PCR products were purified with a QIAquick kit (Qiagen, Valencia, Calif.) according to a protocol supplied by the manufacturer. The purified products were sequenced at the Nucleic Acid Facility at The Pennsylvania State University, University Park, Pa. The *pdhB* sequence obtained from *S. aureus* ATCC 12600 has been deposited in GenBank (accession no. AF235026).

Carbon source consumption per unit of biomass as a function of growth medium a_w . Cultures (100 ml) were grown at 37°C in DFM and in DFM containing NaCl at a_w values of 0.945 and 0.910. Glucose was the only utilizable carbon source and was present at a concentration of 0.5% (wt/vol). Aliquots (0.5 ml) were removed at various time points between 10 and 175 h of incubation, and cells were harvested by centrifugation ($10,000 \times g$ for 10 min). Glucose concen-

trations in the spent medium as well as in uninoculated media were measured with a Sigma Diagnostics glucose oxidase kit according to the manufacturer's instructions. The cell pellet was washed with 0.5 ml of 100 mM sodium phosphate buffer (pH 6.6) and subsequently digested in 1 M NaOH at 90°C for 10 min. The protein content of the digested cell pellets was determined according to the modified Lowry method of Daniels et al. (15), which is suitable for alkaline cell digests. The decrease in glucose concentration in the spent media compared to that in uninoculated medium was expressed on a per-milligram-of-cellular-protein basis to give an estimate of glucose consumed per unit of biomass produced.

RESULTS AND DISCUSSION

Synthesis of several membrane-associated proteins of *S. aureus* is induced when cells are grown in the presence of high concentrations of NaCl. Substantial changes were observed in the membrane-associated protein profile of *S. aureus* when the a_w of the growth medium was reduced by the addition of NaCl. For most proteins where a change could be observed, the protein level decreased with decreasing a_w , as judged by gel spot density. In a few cases, however, a dramatic increase in spot density could be observed (Fig. 1). Indeed, the relative abundance of four spots (no. 15, 33, 72, and 90) increased from a total of 9% in the absence of osmotic stress to more than half (51%) of the total membrane-associated protein visible on the gels at an a_w of 0.89 (Table 1). Of these, proteins 72 and 90 were the most clearly separated from neighboring spots and could be isolated from the gels with relative ease. They were therefore selected for further analysis. Additional gels (not shown) from cultures grown in the presence of high levels of sucrose or glycerol indicated that the increase in the levels of proteins 72 and 90 in response to osmotic stress was humectant specific, as their relative abundance was essentially unaltered compared to that of nonstress controls (data not shown).

Proteins 72 and 90 share homology with the PdhA and PdhB subunits, respectively, of the PDHC. N-terminal sequencing of protein 90 yielded the sequence QMTMVQAIN starting at amino acid 2. A search in GenBank (12) with BLASTp (6) indicated a high degree of similarity to the N-terminal region of the PdhB proteins (the β subunit of pyruvate dehydrogenase) from *B. subtilis*, *Bacillus stearothermophilus*, and *Mycobacterium tuberculosis* (Table 2). In light of this result, the putative *pdhB* gene of *S. aureus* was PCR amplified and sequenced. The predicted amino acid sequence (GenBank accession no. AF235026) corresponded to a polypeptide of 35.2 kDa with a pI of 4.65, consistent with the mobility of protein 90 on 2D PA gels (Table 1). Furthermore, the predicted N-terminal sequence of the *S. aureus* PdhB protein was found to be identical to that determined by N-terminal sequencing of spot 90 beginning at amino acid 3, providing further evidence that spot 90 does indeed correspond to the *S. aureus* PdhB protein. The fact that amino acid identity began at residue 3 also indicates that the N-terminal methionine of PdhB is cleaved off posttranslationally.

PdhB is one of four subunits of the PDHC. Although neither PdhB nor any other PDHC component is an integral membrane protein, the PDHC has previously been reported to be isolated from the membrane fractions of *S. aureus* and *B. subtilis* (1, 2, 3, 19, 20), consistent with our finding in the present paper. Thus, it was reasonable to expect that the levels of the other subunits of this complex also should increase within cells grown at elevated osmolarity and be visualized on

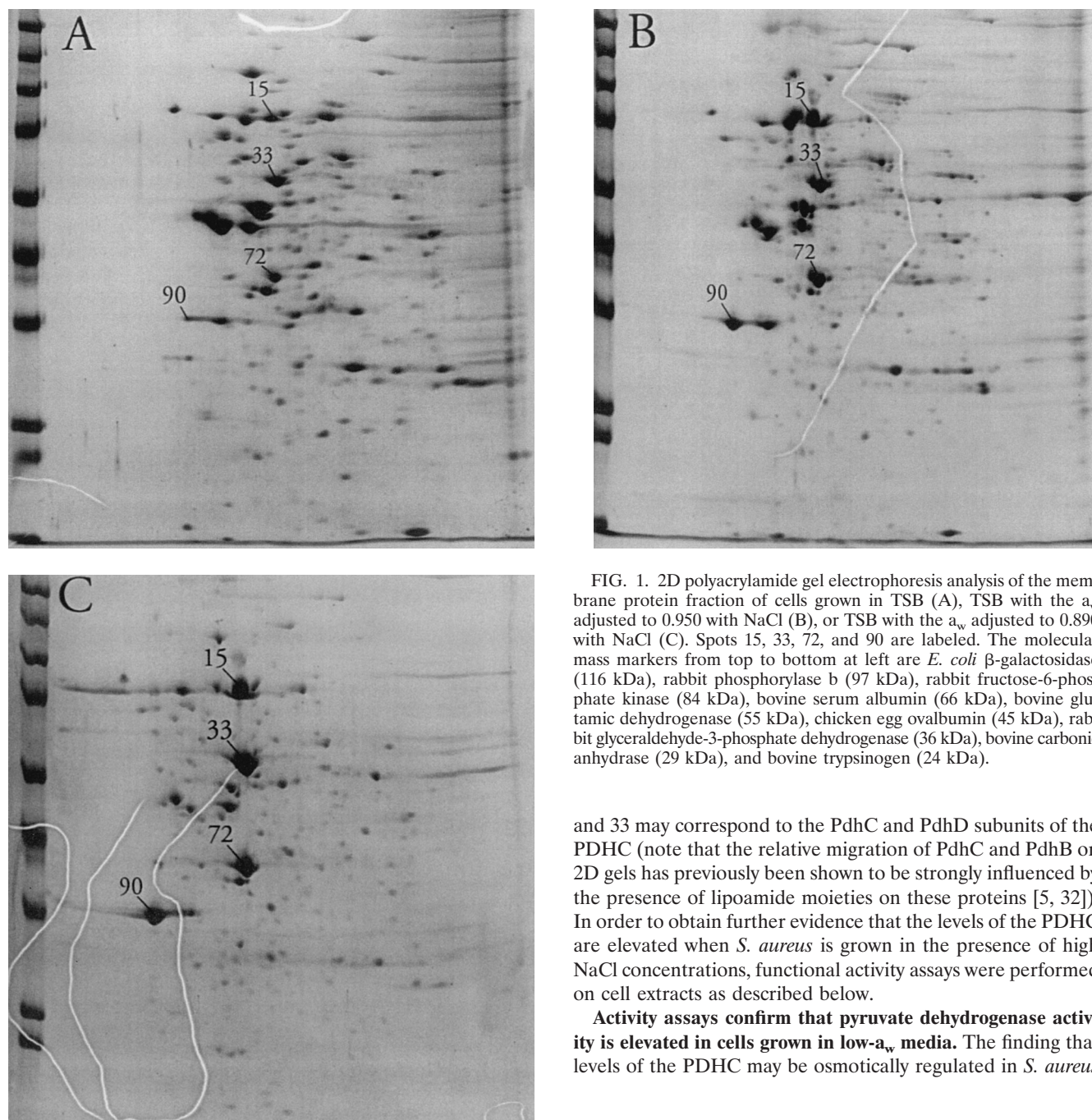


FIG. 1. 2D polyacrylamide gel electrophoresis analysis of the membrane protein fraction of cells grown in TSB (A), TSB with the a_w adjusted to 0.950 with NaCl (B), or TSB with the a_w adjusted to 0.890 with NaCl (C). Spots 15, 33, 72, and 90 are labeled. The molecular mass markers from top to bottom at left are *E. coli* β -galactosidase (116 kDa), rabbit phosphorylase b (97 kDa), rabbit fructose-6-phosphate kinase (84 kDa), bovine serum albumin (66 kDa), bovine glutamic dehydrogenase (55 kDa), chicken egg ovalbumin (45 kDa), rabbit glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), and bovine trypsinogen (24 kDa).

and 33 may correspond to the PdhC and PdhD subunits of the PDHC (note that the relative migration of PdhC and PdhB on 2D gels has previously been shown to be strongly influenced by the presence of lipoamide moieties on these proteins [5, 32]). In order to obtain further evidence that the levels of the PDHC are elevated when *S. aureus* is grown in the presence of high NaCl concentrations, functional activity assays were performed on cell extracts as described below.

Activity assays confirm that pyruvate dehydrogenase activity is elevated in cells grown in low- a_w media. The finding that levels of the PDHC may be osmotically regulated in *S. aureus*

2D gels of membrane protein fractions. Indeed, we were able to obtain the N-terminal sequence for spot 72 (PKLQAQFDA) and found it to exactly match the predicted *S. aureus* PdhA sequence (beginning with amino acid 3) identified from genome sequence data provided by the TIGR website. The relative migration of spot 72 on 2D gels is also consistent with the theoretical M_w (41,400) and theoretical pI (4.9) for the *S. aureus* PdhA protein as predicted from the TIGR sequence data. Due to a lack of sufficient purified protein, we were unable to determine the N-terminal sequences for protein spots 15 and 33; however, the possibility remains that spots 15

TABLE 1. Relative increase in levels of protein spots 72, 90, 15, and 33 as a function of a_w

Spot	Relative abundance ^a at a_w of 0.996, 0.95, 0.89	Apparent M_w (in thousands) ^b	Apparent pI ^b
72	2, 4, 11	40	5.5
90	1, 4, 11	36	5.0
15	2, 4, 13	70	5.5
33	4, 6, 16	57	5.5

^a The relative abundance of each protein spot is expressed as a percentage of the total protein detected on a representative 2D PA gel by scanning densitometry as described in Materials and Methods. Cells were grown in TSB medium adjusted to an a_w value of 0.996, 0.95, or 0.89 by the addition of NaCl.

^b The apparent M_w and pI for each protein spot were estimated by comparing mobilities in 2D gels to a variety of protein standards.

TABLE 2. Homology of bacterial protein sequences to the protein spot 90 N-terminal sequence

Organism	Polypeptide	N position ^a	Sequence ^b	GenBank accession no.
<i>Staphylococcus aureus</i>	Protein 90	2	QMTMVQAIN	AF235026
<i>Bacillus stearothermophilus</i>	PDHC-E1 β ^c	3	QMTMVQAI	X53560
<i>Bacillus subtilis</i>	PDHC-E1 β	3	QMTMIQAI	M57435
<i>Mycobacterium tuberculosis</i>	PDHC-E1 β	26	LTMVQAIN	Z95556
<i>Edwardsiella tarda</i>	Unknown	73	TMVEAIN	L43071
<i>Bacillus cereus</i>	Hemolysin BL	137	TMVEAIN	L20441
<i>Helicobacter pylori</i>	Unknown	244	EMTMVQKI	AE000542
<i>Pseudomonas putida</i>	BCKDHC-E1 β ^d	6	MTMIQA	M57613

^a The position of the sequence shown with respect to the protein N terminus (*S. aureus*) or to the putative translation start site (all others).

^b Obtained by N-terminal amino acid sequencing (*S. aureus*) or deduced from gene sequence (all others).

^c PDHC-E1 β , PDHC, enzyme 1, subunit β .

^d BCKDHC-E1 β , branched-chain keto acid dehydrogenase multienzyme complex, enzyme 1, subunit β .

was somewhat surprising, because to our knowledge, enzymes of primary metabolism have not previously been implicated in the osmotic stress response in any bacterial species. Thus, in order to obtain more definitive evidence, pyruvate dehydrogenase activity assays were performed on cell extracts derived from cultures grown in TSB media at different a_w values. Results were in good agreement with the 2D gel results reported above and indicated that levels of PdhB were approximately three to four times higher in cultures grown in media adjusted to an a_w of 0.95 with NaCl than in nonstress controls (Table 3). It is noted that pyruvate dehydrogenase activity levels within extracts from cultures grown in the presence of Na₂SO₄ or KCl were not significantly different from levels found within extracts derived from cultures grown in the presence of NaCl at the same a_w value (data not shown), suggesting that the increased level of enzyme activity occurred in response to osmotic stress and was not due to a specific response to the Na⁺ or Cl⁻ ion.

Increased pyruvate dehydrogenase activity may provide *S. aureus* with a strategy to meet increased energy demands resulting from osmotic stress. Pyruvate dehydrogenase is a central enzyme of the primary metabolism of *S. aureus*. Therefore, its apparent induction by osmotic stress brought to mind the

common but, with two notable exceptions (16, 21), largely untested assumption that osmoadaptation is an energy-consuming process (17, 27). The rationale behind this assumption is that the maintenance of very steep humectant and compatible-solute concentration gradients across the cytoplasmic membrane (which is necessary if the cell is to maintain its volume and turgor without accumulating high intracellular concentrations of a humectant that may be disruptive to the cell's metabolism) requires substantial activity of the cell's transport systems, which will, directly or indirectly, consume large quantities of ATP. The findings of the present study provided a compelling reason to test this hypothesis. Indeed, we observed that *S. aureus* consumes greater amounts of its carbon and energy source per unit of biomass produced when it is growing under conditions of osmotic stress. Cells growing in DFM with NaCl-adjusted a_w values of 0.996, 0.945, and 0.910 consumed 1.8 ± 1.6 , 5.4 ± 1.1 , and 12.9 ± 2.6 mg of glucose per mg of protein, respectively. This effect appears quite substantial, with consumption of glucose per unit of biomass produced increasing approximately sevenfold when cells are grown at an a_w of 0.910 compared to when they are grown with no stress (a_w , 0.996). The only other studies of which we are aware that directly address energy requirements in relation to osmotic stress were conducted on the relatively nonosmotolerant bacteria *Escherichia coli* (21) and *Klebsiella pneumoniae* (16). While the former study concluded that low water activity did not impose a "large energetic burden" on *E. coli*, the latter study revealed an approximately 10-fold-higher level of carbon source consumption by *K. pneumoniae* per unit of biomass when cells were grown at an a_w of 0.970 (compared to when cells were grown in the absence of osmotic stress). It may be worth noting that an a_w of 0.970 is very restrictive to the growth of *K. pneumoniae*, yielding a growth rate of less than 10% of that observed at an a_w of 0.996 (16), whereas an a_w of 0.910 is only moderately inhibitory to *S. aureus*, yielding a growth rate of approximately 20 to 25% of that observed at an a_w of 0.996 in complex media at 37°C (O. Vilhelmsson and K. J. Miller, unpublished data). It is also worth noting that in addition to this increased carbon source consumption, *S. aureus* has been shown to decrease production of most extracellular proteins in response to osmotic stress (40), which should further increase the energy available for growth and maintenance. Furthermore, extracellular proteins were not included in our biomass estimates, and therefore the glucose consumption per

TABLE 3. PDHC activity and protein spot 90 levels in cell extracts from cultures grown at different a_w values

a_w ^a	Protein 90 level ^b	PDHC activity ^c
0.996	1.67	1.6 ± 0.3
0.97	ND ^d	3.3 ± 0.7
0.95	6.23	4.9 ± 1.0
0.94	ND	6.8 ± 1.0
0.89	9.17	ND

^a Cells were grown to the exponential phase in TSB medium containing various amounts of added NaCl to bring the a_w of the medium to the indicated value.

^b The relative level of protein spot 90 on representative 2D PA gels was determined by scanning densitometry and is expressed in arbitrary volume units. Each gel was loaded with 150 μ g of total membrane-associated protein.

^c The measured ¹⁴CO₂-generating activity (nanomoles of CO₂ per milligram of protein per minute) was found to be dependent on the presence of NAD in the assay buffer (data not shown), eliminating the possibility that other pyruvate-metabolizing enzymes (such as pyruvate decarboxylase) were responsible for all or part of the measured activity and indicating that the activity was carried out by an intact PDHC (since the NAD-consuming reaction is performed by PdhD). Values shown are averages obtained from duplicate measurements from at least two replicate experiments \pm standard deviations.

^d ND, not determined.

TABLE 4. PDHC activity in cell extracts from exponentially growing cultures in media with a_w adjusted with various humectants^a

Humectant	a_w	PDHC activity ^b	C source ^c
Sucrose	0.94	0.86 ± 0.4	Yes
Glucose	0.94	<0.1	Yes
Mannose	0.95	0.77 ± 0.07	Yes
Fructose	0.94	<0.1	Yes
Glycerol	0.95	1.1 ± 0.2	Yes
PEG 8000	0.96	2.6 ± 0.4	No
Glycine betaine	0.94	4.7 ± 0.5	No

^a Cells were grown in TSB media.

^b PDHC activity is defined in footnote c of Table 3, where the activity value (1.6 ± 0.3) for non-osmotically stressed cultures (a_w , 0.996) is also given. Values shown are averages obtained from duplicate measurements from at least two replicate experiments ± standard deviations.

^c Experimentally determined as the presence (yes) or absence (no) of growth of *S. aureus* in DFM with the indicated humectant added as the only available carbon source at a concentration of 0.5% (wt/vol).

unit of biomass, particularly at high a_w values, is probably overestimated. Consequently, the increase in consumption at low a_w values reported herein is probably an underestimate.

Presumably, the apparent increase in energy requirements reported above reflects the cell's efforts to maintain very steep humectant and compatible-solute concentration gradients across the cell membrane. It would therefore be expected that a humectant that is permeant and compatible with cellular metabolism would not lead to increased energy requirements and, hence, increased levels of the PDHC (since other compatible solutes would not be accumulated). Glycerol has just such properties (4), and additional experiments revealed that growth in the presence of high glycerol concentrations (a_w , 0.95) did not lead to increased levels of the PDHC in *S. aureus* (Table 4). Glycerol is utilizable as a carbon source by *S. aureus* (47), and thus, it could be argued (especially in light of our results with sugars as humectants, discussed below) that the low PDHC activity in the presence of glycerol is the result of catabolite repression rather than low energy demands. However, glycerol is highly permeant towards biological membranes (4) and thus probably enters the cells via passive and/or facilitated diffusion. It is therefore unlikely to induce expression of any phosphotransferase system which was shown by Smith et al. (35) to be required for catabolite repression of enterotoxin A production in this organism. However, based on these results, we were prompted to examine the effects of other carbon sources on PDHC activity.

PDHC activity may be subject to catabolite repression. The effects of a variety of nonionic humectants on pyruvate dehydrogenase activity were examined. For these experiments, cells were grown in the presence of high concentrations (a_w adjusted to between 0.94 and 0.96) of several metabolizable carbon sources (sucrose, glucose, fructose, and mannose) as well as two nonmetabolizable humectants (PEG 8000 and glycine betaine). Consistent with an osmotic effect, pyruvate dehydrogenase activity was found to be elevated within extracts derived from cells grown in the presence of high concentrations of PEG 8000 and glycine betaine (Table 4). However, this was not found to be the case when cells were grown in the presence of high concentrations of metabolizable carbon sources. Indeed, levels of pyruvate dehydrogenase activity found within cells grown in the presence of high concentrations of sucrose, glu-

cose, fructose, and mannose were even lower than levels present within nonstress control cultures. This reduction in activity was particularly dramatic in the case of glucose and fructose, suggesting that the levels of the PDHC may be regulated through catabolite repression. Attempts to use nonmetabolizable sugars (D-xylose, D-arabinose, and L-arabinose) as humectants were unsuccessful, as no growth occurred in media adjusted to a_w values of 0.94 or 0.96 with these sugars. To our knowledge, this study is the first to show a link between osmotic stress responses and catabolite repression in *S. aureus*. In light of the effect of osmotic stress on cellular energy demands, discussed above, such a link would seem likely to exist. Interestingly, it may be noted that Ueguchi et al. (41) have provided evidence for a link between stress responses and catabolite repression in *E. coli*. Specifically, these researchers have shown that catabolite repression has a role in the regulation of the *E. coli* RpoS sigma factor.

Concluding remarks. Although the effects of low a_w on the growth of *S. aureus* have been studied for decades, the literature is surprisingly scant on the effects of different humectants on the rate of growth of this organism. Indeed, most growth studies utilizing more than one humectant have primarily focused only on identifying the minimum a_w required for growth (14, 24, 28). However, available data reveal that the growth rate of this food-borne pathogen is, in fact, lower when carbohydrates are used as humectants than when salts are used in this capacity (22, 33, 44a). This effect becomes particularly pronounced at very low a_w values (44a). To our knowledge, there have been no prior attempts to explain this observation, but the results of the present study may provide important insight in this regard. Specifically, increased levels of PDHC may represent an important osmotic stress response for this organism and provide it with a strategy for meeting the increased energy demands associated with maintaining high levels of intracellular compatible solutes. If, however, the osmotic upregulation of the PDHC is counteracted by catabolite repression when metabolizable carbohydrates are used as humectants, it may be predicted that the growth rate of *S. aureus* should be substantially reduced.

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