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A sensor for intracellular ionic strength

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Published in:
 Proceedings of the National Academy of Sciences

DOI:
[10.1073/pnas.0603871103](https://doi.org/10.1073/pnas.0603871103)

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Document Version
 Publisher's PDF, also known as Version of record

Publication date:
 2006

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Citation for published version (APA):

Biemans-Oldehinkel, E., Mahmood, N. A. B. N., & Poolman, B. (2006). A sensor for intracellular ionic strength. *Proceedings of the National Academy of Sciences*, 103(28), 10624 - 10629. DOI: 10.1073/pnas.0603871103

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CBS1

	H1	T1	
	CTSCCSCCBCTTCC	HHHHHHHHHHHTCSBBBBBC	TTCCBBBBBBHHHHHHHHH
OpuA-L1	-NIMIPALTTNIIVN	-GPSVALIKMKTIEVSSLMAVC	--KKSQFICVVTSSQAIARSK : 331
ProV-Ec	--RRTPNGLIRKTPGFGP	RSAALLQDEBDEYGYVIE	--RGNFVGAVSIISL--KTA : 333
OpuCA-Bs	-MNPTP-VTVSALWT	--LSQAIQLMREKRVESLLVVC	--RQNVLKGYYIVVMI--DQN : 305
ProV-Af	IFMISAEIYLIQN---	MEVAIALKEFRKKG-VEVGF	----LISGFAVFLNIIA--- : 304
ProV-Mb	SVMKRPK-PLVSINS-	GPRVAIQLMKIDYGISSIIYVVG	-RNRHLKILMISDA--LEA : 333
OtaA-Mp	NVMKEPK-FVVSLS-	GLOVALLMKXHGIISSIIYVVE	--KAKFLQGFLLVDA--VBA : 329
TM0935-Tm	--KLISLPTVV	EEDTPIEEIVDRILEDVPTVTVYVA	--RDNKLVGMIFVMHLLRVSG : 61
IMPDH-Sp	NGVIIIP-FF-LTP	HKVSEAEELMQSYISGVPIVETLANSKLVGIITN	SMFISD : 150
CLC1-Hs	-CI-MV	GVVVFVSASYTYGELTLLQTTVITLPLVSK	CSMILLGSVERSELQALLQ : 662
AMPKγ2-Hs	--VPTSS	LVVFTTLQVKKRAFFALVANGVRAAPLWESKQ	QS-FVGMILTITFINILH : 328
CBS-Hs	-SLGLSAPLT-VLPTIT	CGHTIILSEKGFQAPVVDEAGV--ILGMVTLGNMLSSLL : 469	

CBS2

	CTSCCSCCBCTTCC	HHHHHHHHHHHTCS	BBBBB	CTTCCBBBBBBHHHHHHHHH	
OpuA-L1	IV-MTTVGTVSKEML	-----VILPIIYCAPTP--LAVVC	--NGFLKGVLIKGSVLE	GALA : 390	
ProV-Ec	GLDAA-LIQAPLAVLAQT	----PLSELLSHVGOAPCA-VPVVD	--ELQQYVGIISKGMLLRALD	: 393	
OpuCA-Bs	IVGDV-YKSHIYTVQNGA	----LLEDTVRILIQGIVVPPVVD	--RQNHLAGIVTRASLVIVY	: 366	
ProV-Af	GIISE-YAEVVSFAFSCS	----LVSALLLRESGEF--VGVVE	--RGEVAGVLLSNEILMLI	: 361	
ProV-Mb	-SLEAAMFPTAPHVAFDR	----PLSEVIPLIASESQYPLAVVND	---SGALLGIIVRGNVLGALA	: 393	
OtaA-Mp	-TMEDVIIKSEFKTTVDT	----PLQDLISIAETIQP-IAIVN	--RNLKLVGVVVGSGSILAALK	: 384	
TM0935-Tm	ANNASEIMLIPVYVHMDT	----PLEEALMIMIDNNIQMPVVD	--RGEIVGILNSLEILLALW	: 140	
IMPDH-Sp	HMTSEHLVTAAVGTLETA	---SILHEHTEKLLPL-VNSGR	----LSGLITIKDIEVIE	: 211	
CLC1-Hs	CCIDQSPFQLVEQTTLHNT	---HTLFSLLGLHLAYV--TSMGK	----LEGLVLALEELQAIK	: 871	
AMPKγ2-Hs	ETFKELVNISPDAASLFDV	---YSLIKN-NIHLPLVITPISGN	----ALYILTHKIKILFLQ	: 409	
CBS-Hs	-VIYKQFQIQLTITLGL	LSHILSMDFEALVVHEQIQYHSTGSSQ	QMVFGVVTAIDLLNFVA : 544		

Anionic C-terminus

OpuA-L1	SIPDEDEVSEIEKEREENK	-----	: 408
ProV-Ec	REGVNNG	-----	: 400
OpuCA-Bs	RSIWGDEEQLMTI	-----	: 380
ProV-Af	-----	-----	: 361
ProV-Mb	IAGENEQFGVSNERASEERTSEERTSREKRTSEERTSEERTSEERNISANTFSG	PAEATVVLIGINRGEA	: 457
OtaA-Mp	TTEDEEQVEEVFQ	-----	: 402
ProV-Mb	GPPSISSGAPNAQNNIISNGVSE	SVPTPLATEPLSSEKQPPVVIC	----- : 506

Fig. 7. Alignment of CBS domains in OpuA type of ABC transporters (top six) and other well studied proteins (bottom four). Sequences of a selected number of proteins are shown, including two proteins (IMPDH and TM0935) of which the structure of the CBS domains has been determined; top sequence corresponds to the OpuA system from *Lactococcus lactis*. The e-value for the CBS domains of OpuA was $9.5e^{-14}$ (<http://motif.genome.jp>), justifying its classification as genuine CBS family member. Scores for the significance of CBS domains in the other proteins were also very high (e-values $\ll e^{-10}$). Sequences were chosen to reveal the variation in the anionic carboxyl-terminal end of the proteins (e.g., absent in ProV from *Archaeoglobus fulgidus* and up to 113 residues in ProV from *Methanosarcina barkeri*). Organisms: OpuAA, *Lactococcus lactis* (AAF37878); ProV, *Escherichia coli* (BAB36963); OpuCA, *Bacillus subtilis* (O34992); ProV, *Archaeoglobus fulgidus* (NP_069814); ProV, *Methanosarcina barkeri* (YP_305877); OtaA, *Methanohalophilus portucalensis* (unpublished data); TM0935, *Thermotoga maritima* (NP_228743); IMPDH, *Streptococcus pyogenes* (YP_283220); CLC1, *Homo sapiens* (P35523); AMPKγ2, *H. sapiens* (Q9UGJ0); CBS (=the enzyme cystathionine β-synthase), *H. sapiens* (AAP35818). C, T, H, and B refer to predicted coil, turn, α-helix, and β-sheet, respectively. Shading is as follows: negatively charged residues (red); positively charged residues (blue); conserved nonpolar residues (grey); and highly conserved residues (black).

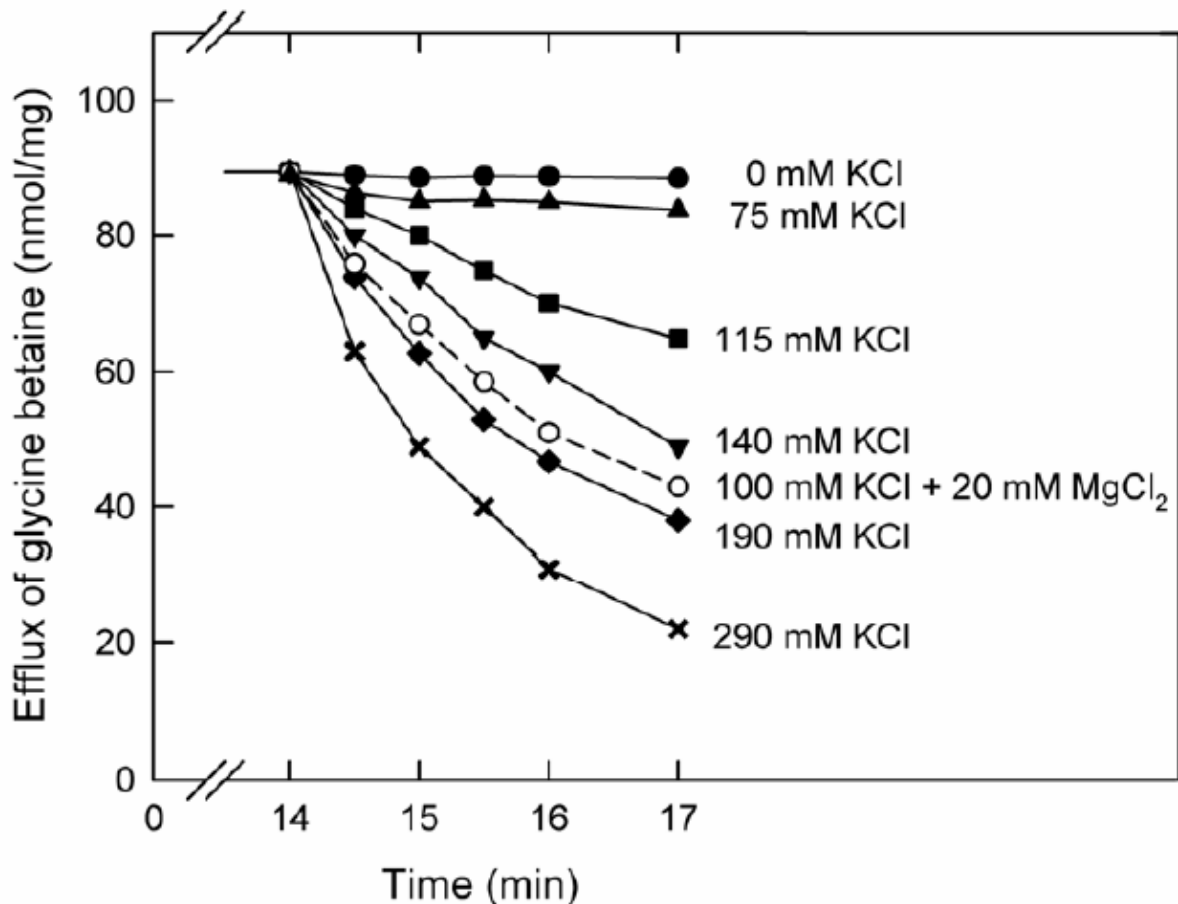


Fig. 8. The effect of K^+ and Mg^{2+} ions on the transport activity of OpuA. ATP-driven efflux of glycine betaine by inside-out reconstituted OpuA was assayed after preloading of the proteoliposomes with [^{14}C]-glycine betaine, essentially as described under "Transport assay" in *Materials and Methods*, except that 50 mM KPi, pH 7.0, plus 450 mM sucrose was used in the external medium. After 10 min of uptake, a plateau in the glycine betaine uptake was reached due to depletion of ATP and buildup of ADP (1). The proteoliposomes preloaded with [^{14}C]-glycine betaine were stored on ice, and aliquots of the mixture were used for individual efflux experiments. For the efflux assay, 45 μ l of the suspension was diluted 5-fold with 10 mM KPi, pH 7.0, to a concentration of 5 mg of lipids/ml and warmed to 30°C for 2 min, after which 20 mM MgATP was added and incubation at 30°C was continued for 2 min. Subsequently, the suspension was diluted 2-fold with prewarmed 10 mM KPi, pH 7.0, plus KCl, MgCl₂, BaCl₂, or other salts at concentrations specified. The proteoliposomes were composed of 40 mol% DOPE, 20 mol% DOPC, and 40 mol% DOPG. The ionic strength of 100 mM KCl and 20 mM MgCl₂ is 0.16, which equals the ionic strength of 160 mM KCl.

1. Patzlaff, J.S., van der Heide, T. & Poolman B. (2003) *J. Biol. Chem.* **278**, 29546-29551.

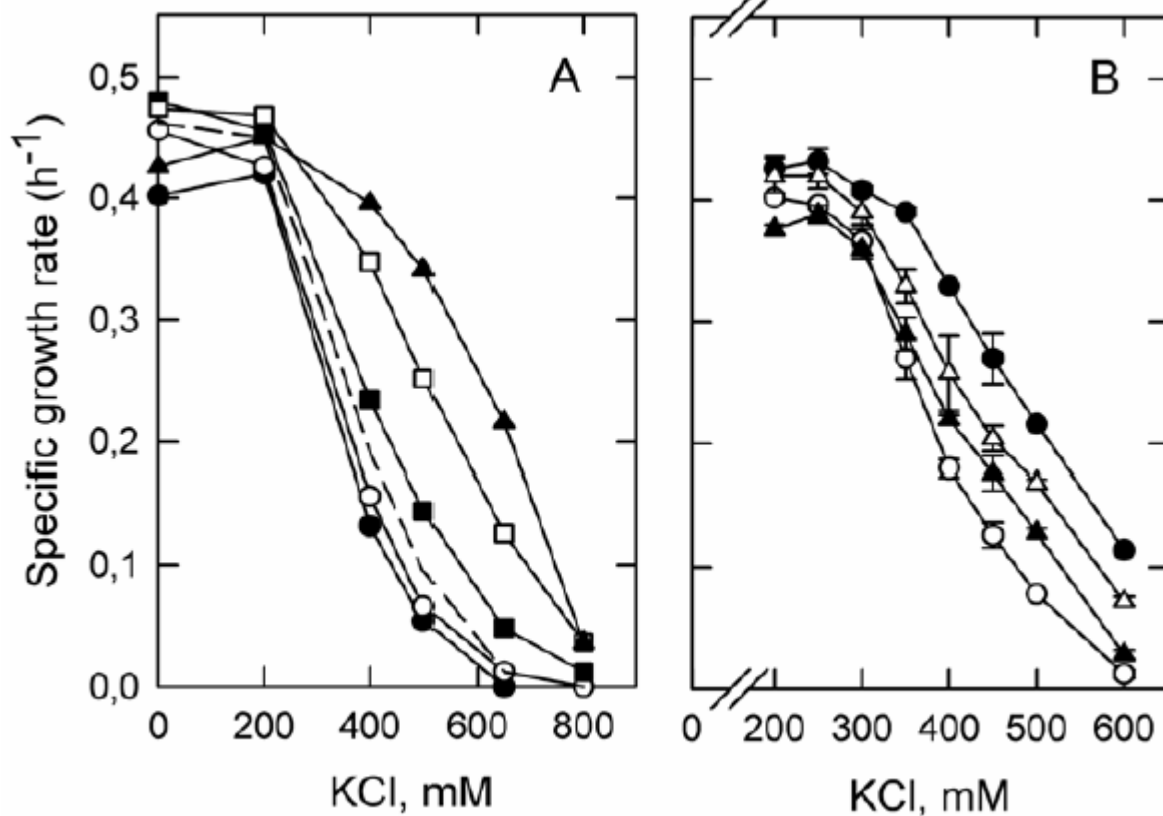


Fig. 9. *In vivo* characterization of CBS mutants. (A) Specific growth rate of *L. lactis* Opu401 carrying pNZOpuAHis (wild-type OpuA) and parent strain NZ9000 carrying control plasmid (dashed line) grown in chemically defined medium with varying concentrations of KCl as indicated. To induce the expression of the *opuA* genes, nisin A was added to the growth medium at 0 (filled circle); 10⁻⁴ (open circle), 10⁻³ (closed square), 10⁻² (open square), or 10⁻¹ % (v/v) (filled triangle). (B) Specific growth rate of *L. lactis* Opu401 carrying pNZOpuAHis, pNZOpuA(Δ12)His, and pNZOpuA(Δ61)His. For the induction of wild-type OpuA (filled circle), OpuAΔ12 (open triangle) and OpuAΔ61 (filled triangle), 10⁻² % of nisin A was used. The strain lacking OpuA is represented by the open circles. The mean and standard deviations of three independent experiments are shown.

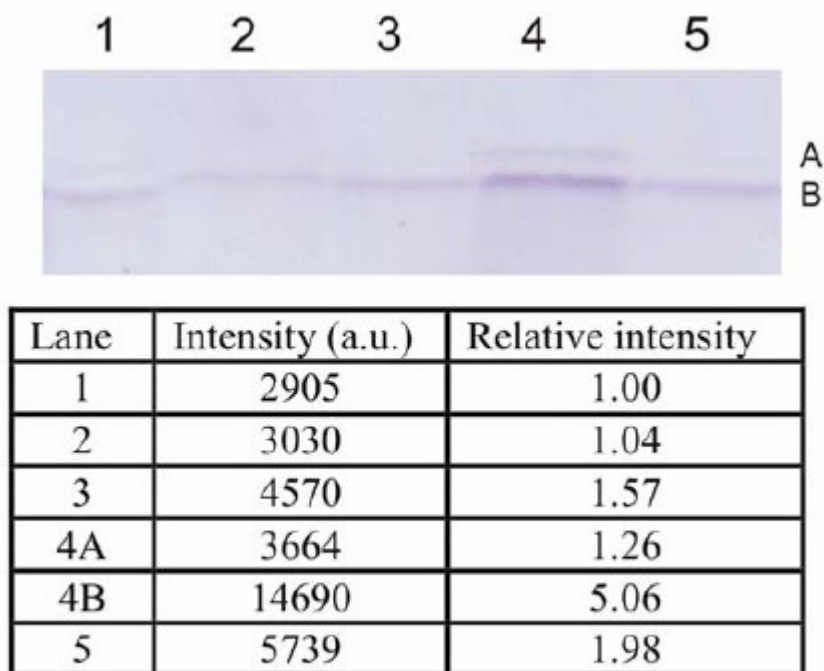


Fig. 10. Physical interaction of CBS domains of OpuA with liposomes. The CBS domains of OpuA were purified as described in *Materials and Methods* and the histidine tag was removed by overnight cleavage with Factor Xa at 20 °C. The cleaved protein was diluted 2-fold with 50 mM KPi, pH 8.0/200 mM KCl and incubated with Ni²⁺-NTA resin (0.25 ml of resin per 1 mg of CBS domain) for 1 hour at 4°C to remove uncleaved protein. The flow-through containing cleaved protein was collected and buffer was exchanged to 20 mM KPi, pH 8.0/20 mM KCl, using NAP10 columns (Amersham Pharmacia Biosciences). Subsequently, 60 µg of purified protein was incubated with 1 mg of liposomes composed of different lipids, 50 mol% DOPE plus 50 mol% DOPC (lanes 2 and 3) or 50 mol% DOPE/12 mol% DOPC plus 38 mol% DOPG (lanes 4 and 5) in 20 mM KPi, pH 6.0, without (lanes 2 and 4) or with 200 mM KCl (lanes 3 and 5). The mixture was incubated for 15 min at 4°C, after which the solution was centrifugated for 25 min at 267.000 × g at 4°C. The pellets, consisting of liposomes with or without pulled-down CBS proteins, were resuspended in denaturation buffer with 4% of SDS and loaded on a 12.5% SDS/PAGE gel supplemented with 1% SDS. Lane 1: background signal after centrifugation from soluble CBS protein incubated without liposomes. In lane 4, some uncleaved protein is visible at position A; B corresponds to the cleaved CBS protein. The intensity of the bands was determined by using IMAGEQUANT (Amersham Pharmacia Biosciences).

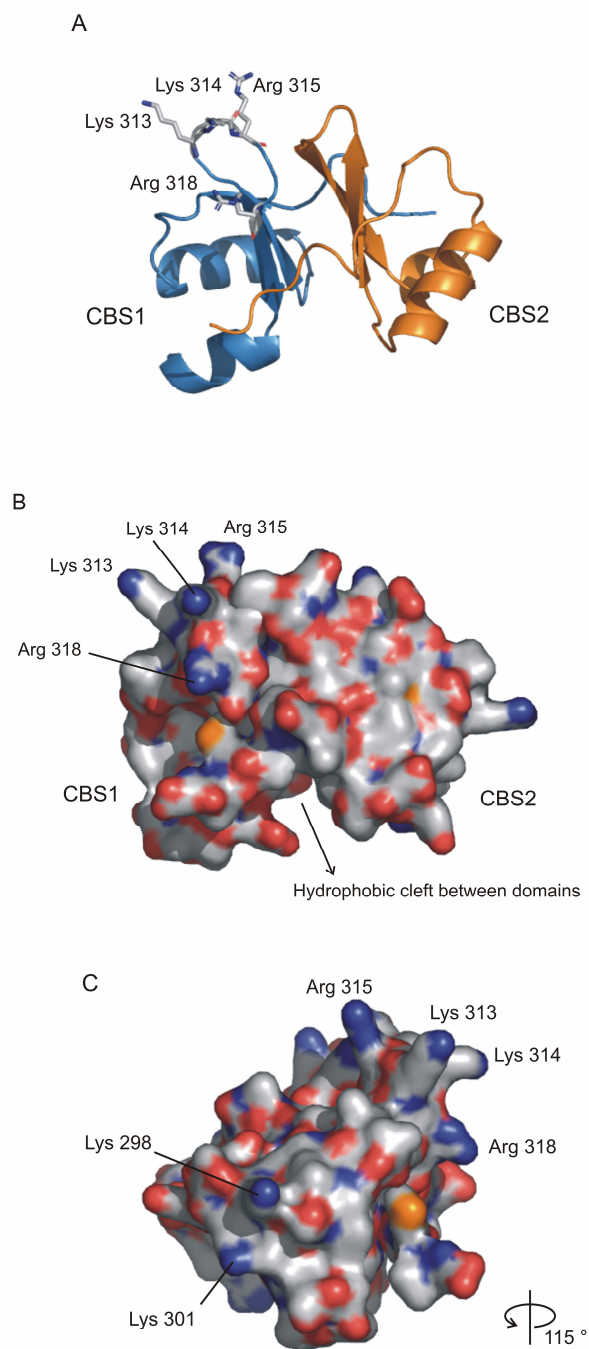


Fig. 11. Model of tandem CBS domain of OpuA (A) and the molecular surface charge distribution (B and C) obtained with the program PYMOL (DeLano Scientific, South San Francisco, CA). The CBS domains were individually modeled on the crystal structure of the CBS tandem Ta0289 from *Thermoplasma acidophilum* [Protein Data Bank (PDB) entry 1PVM], using PHYRE (www.sbg.bio.ic.ac.uk/phyre). The individual domain models were then assembled with reference to the atomic coordinates of the CBS tandem of IMPDH from *Streptococcus pyogenes* (Fig. 1, PDB entry 1ZFJ) to form the tandem CBS pair, using PYMOL. The cationic patches, possibly interacting with the membrane surface, are indicated in B and C.

Table 1. Proteins with one or multiple CBS domains are present in species ranging from bacteria and archaea to humans

Species	Number	Types of proteins with CBS domains
<i>Homo sapiens</i>	50	AMP-PK, CLC, Cys- β -S, IMPDH, PPDE, TP
<i>Mus musculus</i>	45	AMP-PK, CLC, Cys- β -S, IMPDH, PPDE, TP
<i>Arabidopsis thaliana</i>	48	CLC, SIS, TP
<i>Caenorhabditis elegans</i>	28	CLC, IMPDH, TP
<i>Saccharomyces cerevisiae</i>	12	CLC, Cys- β -S, IMPDH, PPDE, TP
Bacteria	5-15	ABC, CLC, CorC, IMPDH, SIS, TP
<i>Escherichia coli</i>	7-8	ABC, CorC, IMPDH, SIS, TP
<i>Lactococcus lactis</i>	5-6	ABC, CorC, IMPDH, TP
Archaea	5-15	ABC, CLC, CorC, IMPDH, MFS, NHA, TP
<i>Halobacterium sp.</i>	12	CLC, CorC, IMPDH, TP
All species	>4,000	

Databases were inspected with a motif-searching algorithm (<http://motif.genome.jp/>). AMP-PK, AMP-activated protein kinases; ABC, ATP-binding cassette transporters; CLC, voltage-gated chloride channels/transporters; CorC, family of putative bivalent cation transporters; Cys- β -S, cystathionine- β -synthase, IMPDH, inosine-5'-monophosphate dehydrogenase; MFS, major facilitator superfamily transporters; NHA, Na⁺/H⁺ antiporters; PPDE, pyridoxal-5'-phosphate-dependent enzyme; SIS, sugar isomerases; TP, proteins with two or four CBS domains in tandem but without catalytic moiety. Proteins with unknown functions and those only found in a limited number species were excluded from the list.

Supporting Materials and Methods

Plasmid construction

pNZOpuAHis(PstI) was constructed by site-directed mutagenesis from pNZOpuAHis, bearing the wild-type *opuAA* and *opuABC* genes (1), using the OpuA(pstI) primer 5'-AGGACGAAGTAGAGGAGATTGCTGCAGAG-GAGGAGAATAAATG-3'; in this construct, the OpuABC protein has a C-terminal 6 histidine tag. This plasmid (unique PstI site at position +1206 with respect to the start codon) was used to construct the different OpuA (deletion) mutants. OpuA Δ 12 was constructed by replacing the *opuAA* gene after digestion with NcoI and PstI for the PCR fragment of the corresponding truncated form of the *opuAA* gene, yielding pNZOpuA(Δ 12)His. The PCR fragment was amplified from pNZOpuAHis with primer pair AALLNcoI 5'-GGGCATGCCATGGCAGTAAAAATAAAAATTG-3' and SAH042 5'-CAGGAACTGCAGCTAAGGCCTCTAAAC-3'. The same strategy was used to construct OpuA Δ 61 and OpuA Δ 119, using primer pair AALLNcoI and SAH041 5'-CGACATCTGCAGTCATCACATCTTTTAATGG-3', yielding pNZOpuA(Δ 61)His, and for pNZOpuA(Δ 119)His primer pair AALLNcoI and SAH040 5'-TAGTAGCTGCAGCTGGAATCATAATATTCTC-3'. To replace the negatively charged C terminus of OpuAA with 10 histidines flanked by Ala, pNZOpuA(Δ 12)His was digested with PstI, and an annealed oligonucleotide, encoding the 10 histidine residues and containing PstI compatible ends, was inserted, resulting in pNZOpuA(Δ 12ins10His)His. Primers used to create the annealed oligonucleotide were EOL0416 5'-GAGCATCACCATCACCATCACCATCACCATCACGCTGCA-3' and EOL0417 5'-GCGTGATGGTGATGGTGATGGTGATGGTGATGCTCTGCA-3'. The tandem CBS pair of OpuA was produced as soluble protein by expressing the corresponding gene fragment (base pairs +763 to +1224) in *L. lactis* NZ9000, using pNZOpuAHis and replacing of the *opuA* genes. All mutants were verified by DNA sequencing; the protein sequence of the C terminus of the various mutants is presented in Fig. 2A.

Deletion of chromosomal opuA genes

An OpuA deletion mutant was constructed in *L. lactis* NZ9000 by homologous recombination. The *opuA* gene replacement vector was generated in the following way: a NcoI-BamHI fragment containing the sequence from position -833 to +49 with respect to the start codon (+1 is first nucleotide of translation initiation codon) of *opuAA* was amplified by using genomic DNA from *L. lactis* NZ9000 and the primers SAH001 5'-AGAATTCCATGGGCATAGCGAGACAAC-3' and SAH004 5'-TTGGCAAACGGATCCAAACCGCATT-3'. A BamHI-XbaI fragment from position +2205 to +2974 (relative to +1 of *opuAA*) was amplified with the primers EOL0426 5'-GAACTGGGATCCAGAAGTTGCGTAAT-3' and EOL0427 5'-CCTTCTATCTAGAATGTCAGAAAGTTTTTTAAG-3'. Both fragments were digested with the corresponding restriction enzymes and ligated into pNZOpuAHis, which is a derivative of pNZ8048. The resulting plasmid, pNZ Δ opuA, was digested with NcoI and XbaI, yielding a 1.65-kb fragment containing both flanking regions in tandem. This fragment was ligated into pORI280 (2), and the resulting plasmid pORI280 Δ opuA was transformed to NZ9000. Because this strain does not contain the essential replication factor (RepA), selection for growth in the presence of erythromycin forces the plasmid to integrate into the chromosome by homologous recombination. Positive colonies were selected on the basis of erythromycin-resistance and a β -galactosidase positive phenotype, using M17 agar plates supplemented with 0.01% X-Gal. A number of the blue colonies were subsequently grown

for ≈ 30 generations under nonselective conditions in M17 medium lacking erythromycin. Nonselective growth allows a second recombination event to occur, which results in the deletion of either the wild-type *opuA* gene or the plasmid pORI280 Δ *opuA*. In both cases, the strains are erythromycin-sensitive and β -galactosidase-negative (white colonies on M17 X-Gal agar plates). A number of clones were selected, and deletion of the *opuA* genes was confirmed by the PCR, phenotype analysis (Fig. 9), and whole-cell transport assay.

Phenotype analysis

Osmotic (salt) sensitivity of *L. lactis* NZ9000 and Opu401 (either or not bearing plasmids for overproduction of OpuA or mutant derivatives) was tested by growing the cells in chemically defined medium (CDM) supplemented with 0.5% glucose/5 μ g/ml chloramphenicol, and different concentrations of salt and nisin. The growth under different conditions, using 96-well plates and a cultivation volume of 300 μ l, was followed in time by measuring the A_{600} .

In vivo experiments

Phenotype of CBS mutants

To study the phenotype and to purify the mutant proteins, the wild-type *opuA* genes (*opuABC* and *opuAA*) were deleted from the chromosome of *L. lactis* NZ9000 by homologous recombination (2), yielding strain Opu401 (see above). The strain with the *opuA* genes deleted displayed severe sensitivity to hyperosmotic stress when compared to the wild-type, that is, when *L. lactis* cells were grown in relatively high osmolality complex broth (not shown). Deletion of the *opuA* genes resulted in a complete loss of the capacity to accumulate glycine betaine, as was confirmed in transport assays; the initial rates of glycine betaine uptake of wild-type and Opu401 strains, both grown in complex broth, were 4.5 and < 0.1 nmol/min \times mg of protein, respectively. With cells cultivated in low-osmolality CDM, deletion of the *opuA* genes had much less effect (Fig. 9A), reflecting the 5- to 10-fold lower level of expression under these conditions (3). Overexpression of the wild-type *opuA* genes in the Opu401 strain and grown in CDM resulted in a dramatic increase in the resistance towards hyperosmotic stress (Fig. 9A). The Opu401/pNZOpuAHis strain could grow up to much higher osmolarities than the parent NZ9000 strain, because of the higher levels of OpuA in the membrane.

The phenotype of the mutants was analyzed by growing *L. lactis* Opu401 transformed by the corresponding plasmids, at varying osmolarities. OpuA Δ 12 and OpuA Δ 61 could be expressed at similar levels in the membrane as wild-type OpuA (Fig. 2B). OpuA Δ 119, on the other hand, showed a normal level of the OpuABC subunit, but the expression of the ATPase subunit (=OpuAA Δ 119) was highly reduced. Fig. 9B shows that, compared to wild-type OpuA, the expression of OpuA Δ 61 only marginally alleviated the sensitivity of *L. lactis* Opu401 to hyperosmotic stress; the expression of OpuA Δ 12 yielded an intermediate phenotype. Clearly, the modifications of the CBS domains in OpuA Δ 12 and OpuA Δ 61 diminish the capacity of the transporter to adjust the intracellular levels of glycine betaine in response to the osmotic stress.

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3. Van der Heide, T. & Poolman, B. (2000) *J. Bacteriol.* **182**, 203-206.