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Sequence analysis and functional studies of a chromosomal region of alkaliphilic *Bacillus firmus* OF4 encoding an ABC-type transporter with similarity of sequence and Na^+ exclusion capacity to the *Bacillus subtilis* NatAB transporter

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Abstract A 14.1-kb DNA fragment was cloned from a lambda library containing inserts of DNA from alkaliphilic *Bacillus firmus* OF4 on the basis of its hybridization to a probe from a previously sequenced alkaliphile homolog of the *natA* gene from *Bacillus subtilis*. Sequence analysis of the entire fragment revealed that, as in *B. subtilis*, the *natA* gene was part of a putative gene locus encoding an ABC-type transporter. In the alkaliphile, the transporter involved three genes, designated *natCAB*, that are part of a larger operon of unknown function. This is in contrast to the two-gene *natAB* operon and to another homolog from *B. subtilis*, the *yhaQP* genes. Like *natAB*, however, the alkaliphile *natCAB* catalyzes Na^+ extrusion as assessed in a mutant of *Escherichia coli* that is deficient in Na^+ extrusion. The full 14.1-kb fragment of alkaliphile DNA sequenced in this study contained several probable operons as well as likely monocistronic units. Among the 17 predicted ORFs apart from *natCAB* were *acsA*, a homolog of a halobacterial gene encoding acetylCoA synthetase; *sspA*, a homolog of a small acid-soluble spore protein; and *malK*, an ATP-binding component that was unaccompanied by candidates for other *mal* transport genes but was able to complement a *malK*-deficient mutant of *E. coli*. No strong candidates for genes encoding a secondary Na^+/H^+ antiporter were found in the fragment, either from the sequence analysis or from analyses of complementation of *E. coli* mutants by subclones of the 14.1-kb piece. There were a total of 12 ORFs whose closest and significant homologs were genes from *B. subtilis*; of these, one-third were in apparently different contexts, as assessed by the sequence of the neighboring genes, than the *B. subtilis* homologs.

Key words Alkaliphile · *Bacillus firmus* · Na^+ extrusion · ABC transporter · *natCAB* · *malK*

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Introduction

Alkaliphilic, nonmarine *Bacillus* species vary with respect to both their Na^+ tolerance and the concentration of Na^+ that is required for growth. However, thus far, all show a requirement for Na^+ to support pH homeostasis, motility, and ion-coupled solute uptake (Krulwich 1995). As yet, no primary Na^+ extrusion systems have been demonstrated in the nonmarine alkaliphiles. By contrast, in marine bacteria (Avetisyan et al. 1991; Dimroth 1994) and in fermentative bacteria (Kakinuma and Igarashi 1995), such systems are apparently important contributors to the transmembrane electrical potential, the $\Delta\Psi$, positive out, when the organisms are growing at elevated pH and the total proton potential is low. Because the extreme alkaliphiles are the extremophiles that are best adapted to the highest pH values, it is of particular interest to determine whether such mechanisms are also used in these "specialists." Experiments on alkaliphilic *Bacillus firmus* OF4 in pH-controlled chemostat cultures on a non-fermentative substrate (malate) indicated that pH homeostasis is the growth rate-determining process as the external pH is elevated to the range of pH 10.6–11.4 (Sturr et al. 1994). Because Na^+ efflux by secondary Na^+/H^+ antiporters is a key limb of the cycle that supports pH homeostasis (Krulwich et al. 1994, 1997), it is quite possible that ongoing loss of cytoplasmic Na^+ through primary extrusion would be detrimental to extreme alkaliphiles unless an inhibitory concentration of Na^+ were present; i.e., primary Na^+ extrusion might be reserved and regulated as a mechanism for Na^+ resistance while alternative mechanisms for enhanced $\Delta\Psi$ generation have been developed for growth in the most alkaline range of pH for growth.

As genes encoding primary Na^+ extrusion systems are identified in other gram-positive bacteria, especially *Bacillus* species, opportunities may arise to clone and study alkaliphile homologs and clarify their roles. Such an opportunity appeared to be presented by the finding of a novel ABC-type extrusion system for Na^+ in *Bacillus subtilis*, the

NatAB system (Cheng et al. 1997). This transport system is part of the ABC-2 group (Reizer et al. 1992) that is encoded by two genes, one of which (*natA*) encodes the ATP-binding hydrophilic subunit; the other (*natB*) encodes an integral membrane protein with a large hydrophilic N-terminal domain followed by a hydrophobic C-terminal domain with six putative transmembrane segments. Presumably, two NatA and two NatB polypeptides are assembled for each transporter complex. The *natAB* genes are expressed together as an operon, and their expression is enhanced by agents such as ethanol and protonophores that compromise the permeability properties of the coupling membrane (Cheng et al. 1997). The phenotype of *B. subtilis* mutants with disruptions in the *natAB* genes indicates that this locus plays no role in pH homeostasis per se but does play a role in Na^+ resistance and K^+ retrieval, especially when ethanol or protonophore is present or when pH is elevated (Krulwich et al. 1994; Cheng et al. 1997).

Of interest in the context of Na^+ extrusion for alkaliphiles was the finding that the gene in the databases with the closest sequence similarity to *natA* was a gene from *B. firmus* OF4 that was sequenced earlier in our laboratory. The gene emerged during a study in which libraries of alkaliphile DNA were used to complement a Na^+/H^+ antiporter-deficient strain of *E. coli* (Ivey et al. 1991). This screen identified the alkaliphile *nhaC* gene. In addition, a second transformant of the mutant *E. coli* strain with substantially elevated Na^+/H^+ antiport activity was found to have a recombinant plasmid that now had a mix of alkaliphile genes and gene fragments including the *natA* homolog; this recombinant plasmid did not complement fresh mutant *E. coli*. Rather, the original complemented strain retained the phenotype when cured of the plasmid. It was presumed to have incorporated part of the original alkaliphile DNA from the recombinant plasmid into its chromosome. That recombination event involved either incorporation of an alkaliphile antiporter or the activation of an antiporter that remained in the *E. coli* mutant. When the *natAB* region of *B. subtilis* was subsequently characterized, it became of interest to examine the possibility that the putative alkaliphile homolog might also be a primary Na^+ extrusion system whose level of expression and effect on pH homeostasis and $\Delta\Psi$ generation at high pH could be examined.

It was also of interest to investigate whether the DNA region in which the alkaliphile *natA* gene was found contained a candidate for an antiporter structural gene. In the current study, a 14.1-kb lambda clone of *B. firmus* OF4 DNA was isolated on the basis of its hybridization with a fragment of the putative alkaliphile *natA*. The sequence includes a probable three-gene, Na^+-excluding, ABC-type transport system of which the *natA* is a component; it does not reveal strong candidates for a secondary antiporter, and contains additional genes of interest, including an *ssp* gene and a gene encoding an ATP-binding protein, that can complement *E. coli* mutants which lack a functional *malK*.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* cells with and without plasmids were grown in LB (Sambrook et al. 1989) or LBK (Goldberg et al. 1987) medium with appropriate antibiotics unless otherwise indicated. *B. firmus* OF4 was grown under the conditions described previously by Guffanti and Hicks (1991).

Preparation and screening of lambda library containing *B. firmus* OF4 DNA

Chromosomal DNA was isolated from *B. firmus* OF4 by standard methods (Ausubel et al. 1992). The DNA was partially digested with *Sau*3AI. Fragments between 10 and 14 kb were purified, treated with bovine calf intestinal alkaline phosphatase (BCIP), ligated with λ-DASH II vector *Bam*HI arms (Stratagene, La Jolla, CA, USA), packaged, and introduced into XL1-Blue MRA (P2 lysogen) cells for plating onto NZY plates as described in the instruction manual. After amplifying and titering, the recombinant λ phage were stored in 7% dimethylsulfoxide (DMSO)-SM buffer (58 g/l NaCl, 20 g/l $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl pH 7.5, 0.01%, w/v, gelatin) at −80°C. The recombinant λ-phage plaques were lifted onto NYTRAN Plus Nylon membranes (Schleicher and Schuell, Keene, NH, USA) and probed with α-^{32}P deoxycytidme triphosphete (dCTP)-labeled polymerase chain reaction (PCR) product from the region of *B. firmus* OF4 DNA that was homologous to *B. subtilis natA*. The primers used for preparation of the PCR product were L_0 (5′-TTCCAGCCATAGAATGGACCA-3′) and R_0 (5′-GACAGTCGAAATGAGGAAGGCC-3′). The positive candidates were identified by standard southern blotting procedures (Sambrook et al. 1989) and then checked by PCR. Rescreening was conducted to yield a single positive plaque. The resulting λ-phage clone was designated as λ-NAT. The λ-NAT was then mapped with multiple restriction enzymes. Five *Eco*RI fragments of 1–7 kb were subcloned into the *Eco*RI site of pBS-SK or pSPT19 vectors. The inserts were sequenced by primer walking on both strands, and additional PCR products were prepared and sequenced as necessary to clarify or confirm the sequence thus obtained. The sequencing employed an Applied Biosystems (Foster City, CA, USA) 373 DNA sequencer at the Biotechnology Center at Utah State University, Logan, UT.

Sequence analysis

The DNA sequences were aligned and analyzed for location of possible open reading frames (ORFs) using the GeneRunner 3.05 program (1994; Hastings Software, Hastings, NY, USA) and GCG software package (Devereux et al. 1984) [Wisconsin Package Version 9.1;

Table 1. Bacterial strains and plasmids

Strains or plasmids	Relevant characteristics	Source or reference
Escherichia coli		
DH5α MCR	F$^-$ *mcrA* Δ*L* (*mrr-hsd, RMS-mcrBC*) Φ*80dlacZ* Δ*M15* Δ (*lacZYA-argF*) U*169 deoR recA1 end1 supE44* λ$^-$ *thi-1 gyrA96 relA1*	Gibco
XL1-Blue MRA	Δ (*mcrA*) *183* Δ (*mcrCB-hsdSMR-mrr*) *173 endA1* supE thi-1 gyrA96 relA1 lac	Stratagene
XL1-Blue MRA (P2)	XL1-Blue MRA (P2 lysogen)	Stratagene
XL1-Blue MRF'	Δ (*mcrA*) *183* Δ (*mcrCB-hsdSMR-mrr*) *173 endA1* supE thi-1 recA1 gyrA96 relA1 lac[F' *proAB lacI^{q}Z* Δ *M15 Tn10*(*Tet^r*)], pBS-SK phagemid host cells	Stratagene
HS2019	F$^-$ *araD*139 Δ*lac* U169 *rpsL thi* Δ *mal E444*	Shuman (1982)
HS3169	F$^-$ *araD*139 Δ*lac* U169 *rpsL* 150 *rel*A1 *deo*C1 *ptsF*25 *flbB*5301 *rbsR* Δ*malK*16 *zjb*729::Tn*10*	Panagiotidis et al. (1993)
DHB4	*araD*139 Δ(*araABOIC-leu*) 7697 Δ (*lac*) X74 *galU gal K rpsl* Δ*phoA* (*Pvu* II) *phoR* Δ *malF3*/F' *lacI^{Q1}*	Boyd et al. (1987)
EP432	*melB* Lid Δ*nha*A:: Km^R Δ*nha*B:: Cm^R Δ *lacZY thr*1	Pinner et al. (1993)
Bacillus firmus		
OF4	Wild type	Guffanti et al. (1986)
Plasmids or phages		
pGEM-3Zf(+)	Cloning vector(Ap^R)	Promega
pSPT19	Cloning vector (Ap^R)	Boehringer Mannheim
pBK36	Shuttle vector (Ap^R in Gram$^-$ Km^R in Gram$^+$)	Cheng et al. (1997)
pYW4	pGEM-3Zf (+) carrying ORF4	This work
pYW5	pGEM-3Zf (+) carrying ORF5	This work
YW54	pGEM-3Zf (+) carrying ORF4 and ORF5	This work
pST1619	pBK36 carrying ORF 16-19 of λ-NAT	This work
pBS-E1	pBS-SK (+) carrying *Eco*RI E1 fragment of λ-NAT	This work
pBS-E2	pBS-SK (+) carrying *Eco*RI E2 fragment of λ-NAT	This work
pBS-E3	pBS-SK (+) carrying *Eco*RI E3 fragment of λ-NAT	This work
pSPT-E4	pSPT19 carrying *Eco*RI E4 fragment of λ-NAT	This work
pSPT-E5	pSPT19 carrying *Eco*RI E5 fragment of λ-NAT	This work
pBS-SK(+) phagemid	Cloning vector (Ap^R)	Stratagene
λ-DASH II phage	Replacing cloning vector(*spi$^-$* red$^+$ gam$^+$)	Stratagene
λ-NAT	λ-DASH II phage carrying a 14.1-kb DNA insert from *Bacillus firmus* OF4	This work

Km, kanamycin; Ap, ampicillin; Cm, chloramphenicol

Genetics Computer Group (GCG), Madison, WI, USA] running on a VAX (Cambridge, MA, USA) 4000–300 computer. The putative ORFs were compared with sequences reported previously in a search of the nonredundant protein database using the FASTA (Pearson and Lipman 1988) and BLAST (Altschul et al. 1990) network service from the National Center for Biological Information. The secondary structure and the free energy calculations of RNA stemloops were analyzed using a network service (http://www.ibc.wustl.edu/zuker/nph-home.cgi). The whole sequence was deposited at GenBank with accession number AF084104.

Assays of $^{22}Na^+$ accumulation by whole cells of *E. coli* EP432

For expression of *B. firmus* OF4 *natCAB* in Na^+-sensitive *E. coli* EP432, ORFs 16–19 from λ-NAT were cloned as a 4.0-kb *Bgl*II fragment. The fragment was isolated from the 7-kb *Eco*RI subclone of λ-NAT and cloned into the *Bam*HI site of pBK36 behind the *ermC* promoter. This recombinant plasmid was designated pST1619 and was transformed into *E. coli* EP432. This transformant and a comparable one with the empty vector were grown overnight in LBK medium. A 1:10 dilution was then made into 45 ml of fresh LBK medium, and growth was resumed for 2 h. The cells were washed once with 50 mM K_2HPO_4·KH_2PO_4–10 mM glucose buffer, pH 7.5. The cells were then resuspended in 90 ml of the same buffer. To start the assay, $^{22}NaCl$ (0.1 μCi) was added to a 5-ml cell suspension to a final concentration of 100 μM, which was stirred continuously. Then, 1 ml of the reaction mixture was filtered at each time point through 0.45-μm HAWP filters (Millipore, Bedford, MA, USA), washed twice with the buffer, dried, and counted by liquid scintillation.

Assays of secondary Na^+/H^+ antiport activity in everted membrane vesicles from *E. coli* EP432

Everted vesicles were prepared from transformants of *E. coli* EP432 as described by others (Pinner et al. 1993). The antiporter activity was measured by a fluorescence assay using acridine orange (Ambudkar et al. 1984). The reaction mixture contained 10 mM Tris-HEPES buffer pH 8.0 plus 140 mM choline–HCl, 5 mM $MgCl_2$. D-Lactate, to 2 mM, was added as an energy source and 10 mM NaCl was added to assay for antiport as Na^+-dependent reduction of the ΔpH established upon D-lactate addition.

Assays for complementation of maltose transport mutants of *E. coli*

The plasmids containing ORF4 (*malK),* ORF 5 (*lrpR*), and ORFs 4 and 5 together were made by cloning PCR products into pGEM-3Zf(+) vector into the *Bam*HI site behind the T7 promoter. The plasmids were designated pYW4, pYW5, and pYW54, respectively. The primers for ORF 4 were orf 4B1 (5′-AGCGGATCCATGGCAGATATTCAATTA-3′) and orf 4SB (5′-AGCGGATCCGCCG AACAACATAC-3′); the primers for ORF 5 were orf 5B1 (5′-AGCGGATCCTTGCAGAAGGATTTTTTGT-3′) and orf 5SB (5′-AGCGGATCCGTTACTGCAGTCA-CATCACC-3′). The primers for ORFs 4 and 5 together were orf 5B1 and orf 4SB. The recombinant plasmids were transformed into *E. coli* strains with mutations in the maltose operon that were obtained from Howard Shuman (see Table 1). These transformants were examined for the ability to grow on M63 agar plates (Miller 1992) containing 0.4% maltose as the carbon source and supplemented with 8 μg/ml each of L-leucine, L-isoleucine, and L-valine, in addition to 1 μg/ml thiamine, as recommended by Dr. Shuman (personal communication).

Results and discussion

Sequence analysis of the lambda clone of *B. firmus* OF4 DNA

The analysis of the *B. firmus* OF4 DNA fragment in λ-NAT indicated a size of 14.1 kb with 39.8% GC content overall. The location of *Eco*RI and *Bgl*II restriction sites and the organization of 20 putative ORFs are shown in Fig. 1 (a and b, respectively). Although designation of likely promoters was not possible throughout the fragment, it is probable that several groups of genes are organized within operons. These include a group of six genes (ORFs 14–19), near the 3′-end of the fragment in which were found the genes (ORFs 16–18, designated *natCAB*) encoding the *natAB* homolog; in the newly sequenced *B. firmus* OF4 homolog, a second predicted ATP-binding component for a transporter, *natC*, is found upstream of the *natAB* genes. The final gene in the putative operon, ORF 19, is very small and has no significant similarity to genes in the database and must therefore be considered tentative. ORFs 14 and 15, however, are larger and similar to other sequenced genes. ORF 15 shows similarity to several regulatory proteins (Rossbach et al. 1994) (Table 2); ORF 14 is a membrane protein that is a candidate for a transporter. Its modest similarity to at least one Na^+-translocating transporter raises the possibility that the operon functions in the regulated, Na^+-dependent transport of a solute and the concomitant reextrusion of the Na^+ by NatCAB. There are two good candidates for promoters upstream of ORF14 [−35 TTGGAA (nt 9043–9048), −10 TATATA (9066–9071); and −35 TTGCAA (9034–9039), −10 TATACT (9057–9062)]; the first of the two possible −10 sequences is identical to that of the *B. subtilis natAB* −10 sequence (Cheng et al. 1997). The first −35 sequence and the entire second, more upstream promoter candidate are similar to the *B. firmus* OF4 *nhaC* promoter (Ito et al. 1997).

The extent of the sequence similarity of these genes to their homologs is shown in Table 2, which summarizes findings of sequences in the databases with the closest sequence similarity to those in the newly cloned region as well as other selected, similar sequences of interest. The *natC* and *natA* gene products are strongly similar to the ATP-binding protein components of other ABC-type transporters. NatA is most similar to an ATP-binding protein that is part of an apparent ABC transporter of as yet unknown function in *B. subtilis*, but it also shows significant similarity to *B. subtilis* NatA. When compared with each other, NatC and NatA exhibited 23% identity and 47% similarity over a 253-aa region. The *natB* gene product is similar to other membrane protein components, including that from *B. subtilis*, which is in an apparent operon with the best match to NatA (Table 2). The gene product designated as NatB also shows modest similarity to *B. subtilis* NatB, 26% identity and 36% similarity, if the gap weighting in the BESTFIT analysis in GCG software package is reduced to 7. The similarity is even more striking when displayed as the hydropathy analysis, according to Kyte and Doolittle (1982) (data not shown). The modest similarity of the whole NatCAB set of gene products to components of an ABC-type bacitracin efflux transport system was notable, but we found no evidence for enhanced bacitracin resistance in *E. coli* strains expressing these genes (data not shown). Perhaps more relevant was the sequence of closest similarity whose function is known, which is a manganese transport system component.

The two ORFs at the ends of the entire DNA insert of the lambda clone were incomplete, but the sequenced regions exhibited significant similarity to known genes from other bacteria, an acetyl CoA synthetase (*acsA*) gene at the 5′-end and a *spo*IIIJ homolog at the 3′-end, which immediately follows the putative operon containing *natCAB* but is likely to be separate. Several additional genes shared strong sequence similarity with known genes from other bacteria. ORF2 is designated *sspA* on the basis of its strong sequence similarity to a gene encoding an α/β-type SASP from *Sporosarcina halophila* (Magill et al. 1990). The alkaliphile gene product has sasp1 (23–32 aa) and sasp2 (41–55 aa) motifs of this type of SASP (Setlow 1992). It represents the second *ssp* gene to be cloned from *B. firmus* OF4, the first one having been a γ-SASP-encoding gene (Quirk 1993). The closer similarity of the *sspA* gene found here to that of another extremophile, a halophile, as compared to the related gene from *B. subtilis* (see Table 2), is worth noting inasmuch as some of the adaptations of the nonhalophilic alkaliphiles, with their somewhat elevated cytoplasmic pH values, may share features with those of the halophiles. Another gene of interest, ORF 4, is shown here to complement a *malK* mutant of *E. coli*, a determination that was made in light of the sequence similarity to ATP-binding proteins of ABC-type transporters for maltose or components, e.g., UgpC, that can substitute for MalK (Table 2) (Hekstra and Tommassen 1993). The *malK* gene may be

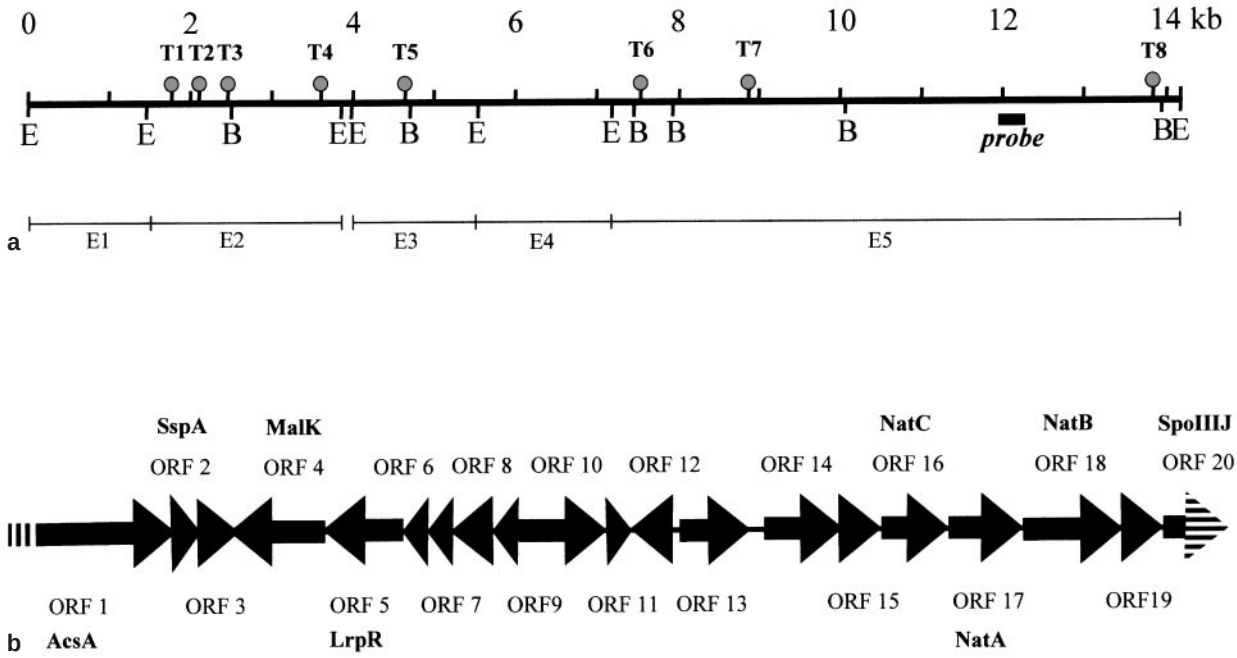

Fig. 1a,b. Diagrammatic representation of the deduced arrangement of ORFs in the 14.1-kb fragment of *Bacillus firmus* OF4 DNA. **a** *T* indicates the putative stem-loop structures. The predicted free energies (Kcal/mole) for T1 to T8 are −21.5, −15.7, −14.6, −23.8, −10.3, −18.1, −19.8, and −12.0, respectively. *E*, *Eco*RI sites; *B*, *Bgl*II sites. The probe that was used for isolation of the λ-NAT clone is shown as a *dark line* in a region of *B. firmus* OF4 that is homologous to *B. subtilis natA*. The *Eco*RI fragments listed in constructs in Table 1 are indicated (*E*1–*E*5); these subcloned fragments were sequenced, together with selected PCR products, and the subclones were additionally used in selected experiments. **b** *Arrows* indicate ORFs and their direction of transcription. The basis for naming some ORFs is discussed in the text and derives from the information shown in Table 2

part of a functional unit with another ORF that has been designated *lrpR* because it is predicted to encode a leucine-rich protein (Table 2). LrpR exhibits sequence similarity in its C-terminal region (found in LrpR between 231 and 291 aa) to a helix-turn-helix type regulatory protein SrmR of *Streptomyces ambofaciens* (Geistlich et al. 1992). We hypothesized that Lrp regulates *malK* expression. The significance of having an independently regulated gene encoding an ATP-binding component of a larger transporter encoded elsewhere on the chromosome is unclear. There is a candidate for a promoter for *lrpR* that is −35 TAGCAAC (nt 4618–4612) and −10 TTGCAGAA (4587–4580); except for the longer spacing, which may be shortened by a stem-loop structure, this is similar to the *B. subtilis* σ^L type promoters that are associated with expression of degradative enzymes (Debarbouille et al. 1991). In addition, at a position that is 32 nt upstream of the ATG translational start proposed for ORF 4, there is a CRE sequence, TGAAAGGGGTTACA (3536–3523), that fits the consensus for such catabolite-responsive elements of gram-positive bacteria (Chambliss 1993; Hueck et al. 1994). Such elements are found in diverse locations relative to the transcriptional and translational starts of the genes with which they are associated (Hueck and Hillen 1995).

Finally, it might be noted that ORF 13 exhibits sequence similarity to an unknown gene in *B. subtilis*, *yhaX* (Table 2). The products of other genes with similarity to these possible homologs are part of a superfamily that includes haloacid dehalogenases and serine phosphate phosphatases (Aravind et al. 1998). The context of ORF 13 in the present clone, i.e., the genes surrounding it, differs from that of the *B. subtilis yhaX* gene. A total of 12 putative genes in the entire sequence have a gene from *B. subtilis* as the closest match in the databases. Of these, the contexts of one-third are clearly different from the contexts of the closest *B. subtilis* match(es) as assessed by the genes surrounding them. Earlier, it was noted that the arrangement of gene clusters relative to one another on the chromosome in *B. firmus* OF4 was often distinct from those in *B. subtilis* (Gronstad et al. 1998).

On the basis of the sequence analysis, there are no strong candidates for genes encoding a secondary Na^+/H^+ antiporter that was anticipated to be in the region of the *natCAB* genes. Possibly one of the ORFs of unknown function is such a gene, nonetheless, and further analysis will reveal this function when those genes are expressed in low copy number in antiporter-deficient mutant strains of *E. coli* or bacilli. Preliminary examination of recombinant pBS-SK and pSPT19 vectors with inserted *Eco*RI fragments of the λ-NAT that were used for sequencing did not show complementation of such mutants of *E. coli*, but a more rigorous examination of single genes under more controlled expression might reveal complementation. It is also possible, however, that the gene in question is in a region

Table 2. Analysis of open reading frames (ORFs) in the 14.1-kb region of *Bacillus firmus* OF4 DNA

ORF	Position	Size of product (amino acids)	Homology to	Function	Identity % (amino acids overlap)[a]	Similarity % (amino) acids overlap)[a]	Species	Acession number of homologs
1	1–1671	556[b]	Acs-4 (642 aa)	Acetyl-CoA synthetase C-terminal portion	46 (546 aa)	64 (546 aa)	*Archaeoglobus fulgidus*	AE001037 (GB)
			Acs-A (572 aa)	Acetyl-CoA synthetase	33 (542 aa)	49 (542 aa)	*Bacillus subtilis*	P39062 (SP)
2	1817–2020	67	Sspl (72 aa)	Small acid-soluble spore protein 1	89 (66 aa)	93 (66 aa)	*Sporosarcina halophila*	Q00213 (SP)
			SspB (67 aa)	Small acid-soluble spore protein B	78 (64 aa)	84 (64 aa)	*Bacillus subtilis*	P04832 (SP)
3	2084–2347	87	None					
4	3490–2390	366	MsmX (365 aa)	Multiple sugar-binding transport ATP-binding protein	72 (365 aa)	84 (365 aa)	*Bacillus subtilis*	P94360 (SP)
			UgpC (356 aa)	*sn*-Glycerol-3-phosphate transport ATP-binding protein	49 (367 aa)	68 (367 aa)	*Escherichia coli*	P10907 (SP)
			MalK (371 aa)	Maltose/maltodextrin transport ATP-binding protein	48 (361 aa)	63 (361 aa)	*Escherichia coli*	P02914 (SP)
5	4488–3598	296	YxkF (297 aa)	Similar to leucine-rich protein	26 (257 aa)	48 (257 aa)	*Bacillus subtilis*	D83026 (DDBJ)
			LrpR (282 aa)	Leucine-rich protein	24 (293 aa)	41 (293 aa)	*Streptococcus equisimilis*	Q54087 (SP)
6	4837–4616	73	Fus/Tls (526 aa)	RNA-binding protein polyG/D/S domain	36 (50 aa)	52 (50 aa)	*Homo sapiens*	P35637 (SP)
7	5123–4890	77	YheE (72 aa)	Unknown	37 (69 aa)	55 (69 aa)	*Bacillus subtilis*	Z99109 (EMBL)
8	5698–5177	173	None					
9	5855–5616	79	YpuC (129 aa)	Unknown	25 (54 aa)	50 (54 aa)	*Bacillus subtilis*	P35152 (SP)
10	5845–6957	370	YheB (377 aa)	Unknown	41 (369 aa)	64 (369 aa)	*Bacillus subtilis*	Z99109 (EMBL)
11	7068–7424	118	YheA (118 aa)	Unknown	59 (114 aa)	81 (114 aa)	*Bacillus subtilis*	Z99109 (EMBL)
12	7668–7471	65	None					
13	7806–8690	294	YhaX (265 aa)	Unknown	46 (251 aa)	69 (251 aa)	*Bacillus subtilis*	Z99109 (EMBL)
			SerB (322 aa)	Phosphoserine phosphatase	34 (63 aa)	51 (63 aa)	*Escherichia coli*	P06862 (SP)
14	9108–9839	243	Aq097 (407 aa)	Unknown	20 (231 aa)	43 (231 aa)	*Aquifex aeolicus*	AE000673 (GB)
			Sgc6 (443 aa)	NADH dehydrogenase (ubiquinone chain 4)	23 (225 aa)	40 (225 aa)	*Leishmania tarentolae*	F30010 (PIR)
			HuNaDC-1 (552 aa)	Sodium-dependent transporter	22 (89 aa)	47 (89 aa)	*Helicobacter pylori*	AE000541 (GB)
15	9870–10229	119	YtrA (130 aa)	Unknown, similar to transcription regulator (GntR family)	35 (98 aa)	59 (98 aa)	*Bacillus subtilis*	AE008220 (GB)
			MocR (493 aa)	Rhizopine catabolism regulation	36 (68 aa)	62 (68 aa)	*Rhizobium meliloti*	P49309 (SP)
16	10273–11145	290	YhcG (232 aa)	ABC transporter ATP-binding protein	28 (216 aa)	54 (216 aa)	*Bacillus subtilis*	P54591 (SP)
			YtrB (292 aa)	Unknown, similar to ABC transporter	28 (229 aa)	51 (229aa)	*Bacillus subtilis*	AF008220 (GB)
			BcrA (306 aa)	Bacitracin transport ATP-binding protein	27 (222 aa)	47 (222 aa)	*Bacillus licheniformis*	P42332 (SP)
17	11212–12111	299	YhaQ (298 aa)	Unknown, similar to ABC transporter ATP-binding protein	61 (296 aa)	79 (296 aa)	*Bacillus subtilis*	Y14078 (EMBL)
			NatA (246 aa)	Na^+ABC transport ATP-binding protein	36 (212 aa)	58 (212 aa)	*Bacillus subtilis*	P46903 (SP)
			BcrA (306 aa)	Bacitracin transport ATP-binding protein	30 (300 aa)	54 (300 aa)	*Bacillus licheniformis*	P42332 (SP)
18	12104–13366	420	YhaP (419 aa)	Unknown, transmembrane protein	47 (421 aa)	66 (421 aa)	*Bacillus subtilis*	O07523 (SP)
			Mj1024 (403 aa)	Unknown, similar to YhaP	25 (400 aa)	47 (400 aa)	*Methanococcus jannaschii*	Q58430 (SP)
			Mth1371 (355 aa)	Unknown, similar to NatB in *B. subtilis*	22 (254 aa)	41 (254 aa)	*Methanobacterium thermoautotrophicum*	AE000900 (GB)
19	13472–13738	88	Unknown					
20	13771–14101	108[b]	SpoIIIJ (259 aa)	N-terminal portion of stage III sporulation protein precursor	53 (96 aa)	74 (96 aa)	*Bacillus subtilis*	Q01625(SP)

aa, amino acids

[a] The identity % and similarity % were based on the BLAST 2.0 (Altschul et al. 1990). The matrix is BLOSUM62, gap existence is 11, and gap extension is 1

[b] Incomplete ORF

nearby that was not encompassed in λ-NAT. Alternatively, the original antiporter activity ascribed to an alkaliphile gene that was incorporated into the *E. coli* mutant chromosome may have resulted from an alkaliphile regulatory gene that upregulated antiporter-encoding *nhaB* or *chaA* in the *nhaA* deletion strain of *E. coli* employed.

Functional studies of *natCAB*

A fragment of λ-NAT that encompassed the *natCAB* genes and a small downstream region (putative ORF19 of unknown function) was cloned into pBK36 as described under "Materials and methods." This plasmid, pST1619, failed to increase the resistance of Na^+-sensitive *E. coli* strain EP432 to concentrations of Na^+ in the range exceeding 250 mM, which can be assayed by growth experiments, in LBK medium at pH 7.5. Nor did isolated membrane vesicles of *E. coli* EP432/pST1619 exhibit an increase over the control in secondary Na^+/H^+ antiporter activity (data not shown). However, when a capacity for Na^+ exclusion was directly measured, the transformant of *E. coli* EP432 exhibited a significantly increased ability to exclude Na^+ over the plasmid control transformant (Fig. 2). In the experiment depicted in Fig. 2, the external concentration of Na^+ was 100 μM. At the 5-min point, the cytoplasmic concentration of Na^+ was 0.52 mM in the control cells and was 0.20 mM in the cells from *E. coli* EP432/pST1619. Taken together, the results are consistent with the expectation based on the sequence analysis, i.e., that this is a primary transport system, presumably coupled to ATP hydrolysis that, like NatAB from *B. subtilis*, extrudes Na^+. The coupling of Na^+ extrusion to H^+ entry via secondary Na^+/H^+ antiporters is a crucial component of the Na^+ cycle that supports pH homeostasis in *B. firmus* OF4, and this cycle requires adequate cytoplasmic Na^+ to support this ongoing antiport activity (Krulwich et al. 1997). Presumably, therefore, this primary Na^+ extrusion system is highly regulated so that its expression is limited to circumstances in which the cytoplasmic $[Na^+]$ greatly exceeds this necessary level for pH homeostasis. It would be of interest to overexpress NatCAB in *B. firmus* OF4 and examine whether indeed this would be adverse to pH homeostasis at highly alkaline pH under growth conditions in which Na^+ concentrations in the medium were moderate.

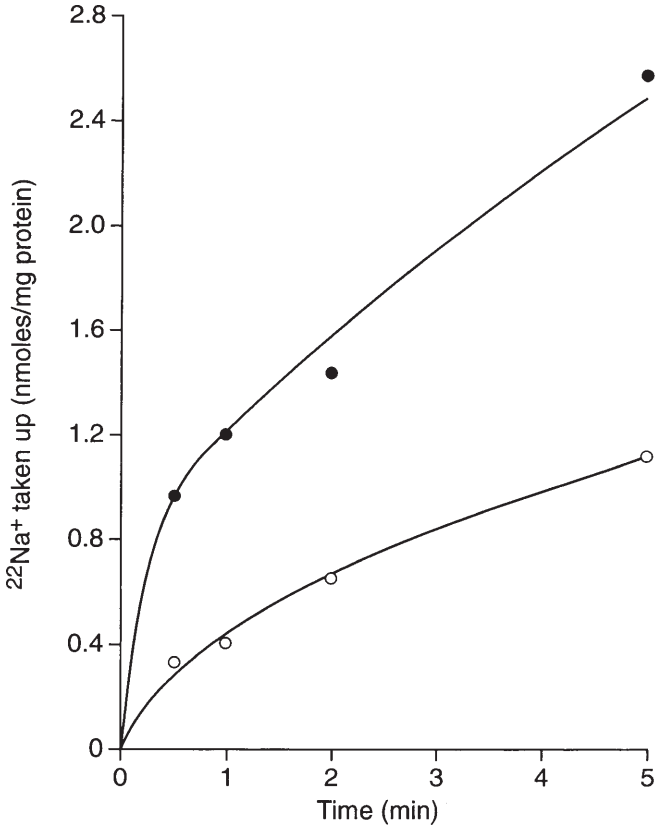

Fig. 2. Accumulation of radioactive Na^+ by Na^+-sensitive *E. coli* strain EP432 transformed with a control plasmid and a plasmid containing *B. firmus* OF4 *natCAB* genes. Logarithmically growing cells of EP432/pBK36 (*solid circles*) or EP432/pST1619 (*open circles*) were harvested, washed, and resuspended as described in Materials and methods. NaCl, to 100 μM, plus carrier-free $^{22}Na^+$ was added to start the reaction, and samples were taken at the times indicated for liquid scintillation counting

Complementation of an *E. coli malK* mutant by the putative *B. firmus* OF4 *malK*

Although not shown, neither *E. coli* HS2019 (Δ*malE*) nor DHB4 (Δ*malF*) was complemented for growth on maltose by pYW4, pYW5, pYW54, or control plasmid pGEM-3Zf(+), as expected given the sequence analysis. On the other hand, *E. coli* HS3169 (Δ*malK*) was complemented both by pYW4 and, somewhat more strongly, by pYW54, but not by either pYW5 or the control plasmid (Fig. 3).

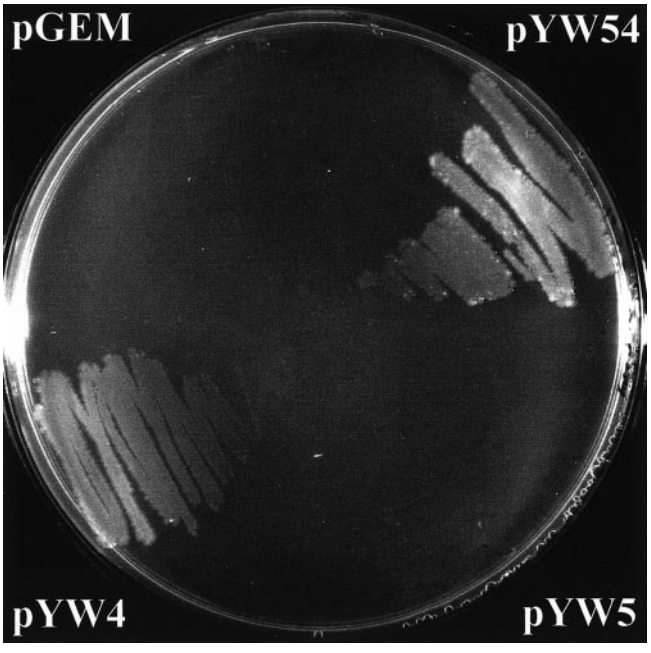

Fig. 3. Complementation of a *malK* mutant of *E. coli* by the *B. firmus* OF4 *malK* gene, *E. coli* HS3169

These results support the designation of ORF4 as *malK* and the hypothesis that ORF 5 (*lrpR*) is a regulatory gene for a putative two-gene operon, *lrpR-malK*. Because there is recognized permissiveness with respect to the substitution of ATP-binding proteins among ABC-transporters with distinct specificities (Ehrmann et al. 1998), the complementation by itself does not support the choice of the *malK* designation. However, the complementation, combined with the sequence similarity, and the presence of a CRE candidate provide significant support.

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