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Biosynthesis of Active *Bacillus subtilis* Urease in the Absence of Known Urease Accessory Proteins

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Bacillus subtilis **contains urease structural genes but lacks the accessory genes typically required for GTP-dependent incorporation of nickel. Nevertheless, *B. subtilis* was shown to possess a functional urease, and the recombinant enzyme conferred low levels of nickel-dependent activity to *Escherichia coli*. Additional investigations of the system lead to the suggestion that *B. subtilis* may use unidentified accessory proteins for in vivo urease activation.**

Urease is a Ni-containing enzyme found in plants, fungi, and bacteria (15). This protein participates in the recycling of environmental nitrogen and serves as a virulence factor in pathogenic microorganisms associated with gastric ulceration and urinary stone formation (22). Most bacterial ureases possess three structural subunits (encoded by *ureABC*) associated into a trimer of trimers [$(\alpha\beta\gamma)_3$], with each UreC subunit containing a dinuclear Ni active site bridged by a carbamylated lysine (4, 16, 28). *Helicobacter* species have only two subunits (UreA, a fusion of the small subunits [β and γ] in other bacteria, and the large subunit, designated UreB) in a $(\alpha_3\beta_3)_4$ macromolecular structure (14). Fungi and plants contain a homohexamer (α_6) of a fusion of the three bacterial sequences (30). Synthesis of active urease requires the action of several accessory proteins (23), with the best-studied system found in *Klebsiella aerogenes*, in which the structural genes are found in a gene cluster containing four accessory genes (*ureDABCEFG*). By use of this system, UreD-UreF-UreG was identified as a GTP-dependent molecular chaperone that binds urease apoprotein (8, 32), while UreE was shown to function as a metallochaperone that delivers Ni^{2+} (11, 25, 31).

Genome sequence analysis has revealed that, in contrast to other ureolytic microorganisms, *Bacillus subtilis* contains only urease structural genes (*ureABC*) and lacks homologues to any accessory genes (18). Despite this dearth of urease genes, the organism exhibits urease activity and grows with urea as the sole nitrogen source unless *ureC* is inactivated (12).

Urease activity in *B. subtilis*. *B. subtilis* SF10 cells (wild type, SMY derivative; from Susan Fisher) (3) were cultured at 37°C in S7 minimal medium (37) plus 0.2% glutamate. A low but detectable level of urease activity (0.113 ± 0.006 U/mg protein, where one unit is the amount of enzyme required to hydrolyze 1 μmol of urea per min at 37°C in 50 mM HEPES buffer, pH 7.8, containing 50 mM urea) (38) was observed in cell extracts obtained by sonication followed by centrifugation (10,000 × *g*, 20 min, 4°C). This level of activity is comparable to the level of 0.103 ± 0.012 U/mg (after correction to the same units) described previously for extracts of these cells (3) and compares to ~2 U/mg for cell extracts of *K. aerogenes* (36) or 2,500 U/mg for the purified *K. aerogenes* urease (35). The addition of 100 μM $NiCl_2$ to the culture had no effect on the urease activity (0.107 ± 0.016 U/mg), which suggests that the trace levels of Ni^{2+} in the minimal medium were sufficient for synthesis of active urease or that Ni^{2+} was not required.

Overexpression of *B. subtilis ureABC* in *B. subtilis* and *Escherichia coli*. To test whether Ni^{2+}-dependent activity is observed in *B. subtilis* that overproduces the urease, pDR-BsABC was constructed by amplifying the *B. subtilis ureABC* genes from pURE91 (a pET23-derived plasmid provided by Susan Fisher), digesting with SalI and NheI, and cloning into pDR111 (7). *B. subtilis* RB247 (*trpC2 pheA1*) (from Rob Britton) containing pDR-BsABC was grown in LB medium supplemented with 100 μg/ml ampicillin, 0.5 mM $NiCl_2$, and in some cases, IPTG (isopropyl-β-D-thiogalactopyranoside). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to analyze the proteins (19) with 15% polyacrylamide running gels and 4.5% stacking gels and Coomassie brilliant blue (Sigma) staining. As shown in Fig. 1A, urease expression was greatly enhanced in the recombinant cells, while SF10 cell extracts exhibited no visible urease proteins. Despite the larger amount of urease protein in cell extracts of the *B. subtilis* transformant, the activity level was lower (0.081 ± 0.026 U/mg) than in the nonrecombinant strain. Growth with 0.5 mM Ni^{2+} resulted in ~3.5-fold-higher level of activity (0.281 ± 0.105 U/mg) than that of nonrecombinant *B. subtilis* SF10. We conclude that urease apoprotein is activated with low efficiency in *B. subtilis*.

Recombinant *B. subtilis* urease was produced in *E. coli* C41(DE3) cells (20) containing pURE91. Cultures were grown in Terrific Broth (TB) (Fisher Biotech) with ampicillin at 37°C to an optical density at 600 nm of ~0.4, induced with 0.5 mM IPTG, and harvested after 14 to 16 h. Urease was highly expressed (Fig. 1B); however, the level of activity measured in cell extracts was very low (0.14 ± 0.02 U/mg). Growth of the *E. coli* transformant in TB medium containing various Ni^{2+} con-

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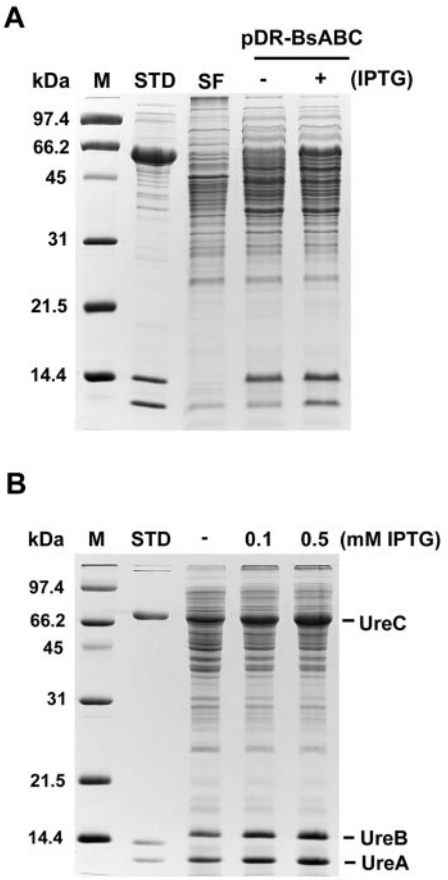

FIG. 1. Expression of recombinant *B. subtilis ureABC* in *B. subtilis* and *E. coli*. (A) Cultures of *B. subtilis* RB247 cells transformed with pDR-BsABC were induced with 0.5 mM IPTG, and the cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes: M, molecular mass markers (for phosphorylase *b*, the M_r was 97,400; for bovine serum albumin, the M_r was 66,200; for ovalbumin, the M_r was 45,000; for carbonic anhydrase, the M_r was 31,000; for soybean trypsin inhibitor, the M_r was 21,000; and for lysozyme, the M_r was 14,400); STD, enriched *B. subtilis* urease standard; SF, cell extracts of SF10 cells grown in S7 minimal medium with glutamate as the nitrogen source; pDR-BsABC − and + (IPTG), cell extracts of the *B. subtilis* transformant. (B) Effects of various IPTG concentrations on expression of recombinant *B. subtilis* urease genes in *E. coli* C41(pURE91) cell extracts. Lanes: M, molecular mass markers; STD, 6 μg of purified *K. aerogenes* urease; −, uninduced control; 0.1 and 0.5 (mM IPTG), extracts of the cells induced with 0.1 mM and 0.5 mM IPTG, respectively.

centrations revealed that urease activity was Ni^{2+} dependent, with maximal activity of 6.4 ± 0.9 U/mg observed when the medium was supplemented with 5 to 7 mM $NiCl_2$ (higher $[Ni^{2+}]$ led to cell toxicity).

Activation of *B. subtilis* urease. We examined whether *B. subtilis* urease in cell extracts could be activated by incubation at 37°C for 90 min in 100 mM HEPES (pH 8.3), 150 mM NaCl, 100 mM $NaHCO_3$, and 300 μM $NiCl_2$. Although these conditions are known to activate ~15% of the *K. aerogenes* urease apoprotein (16, 26, 27), urease activity in nonrecombinant *B. subtilis* extracts decreased 50% and levels of activity in extracts of the *B. subtilis* transformant were unchanged. For extracts from *E. coli* C41(DE2)[pURE91] cells grown with 7 mM $NiCl_2$, activation led to modest increases in activity (<10%). In contrast, for cells grown without supplemental Ni^{2+}, the activity increased from 0.056 to 2.9 U/mg. Mn^{2+} also activated the enzyme (0.42 U/mg), while other metal ions had negligible effects. Notably, Mn^{2+} activates *K. aerogenes* urease apoprotein, yielding ~2% of the activity generated by Ni^{2+} activation (26, 39). The growth studies with various Ni^{2+} concentrations and in vitro activation results both suggest that the *B. subtilis* urease is a Ni-containing enzyme, like all other ureases that have been characterized (15).

Characterization of recombinant *B. subtilis* urease. In order to understand the low level of urease activity in *E. coli* C41(DE3) cells containing pURE91, even when grown with high Ni^{2+} and despite the high-level production of urease subunits, we characterized the properties of the enriched enzyme. Efforts to purify recombinant urease by using ion exchange and hydrophobic interaction chromatography resins, even with potential stabilizing agents, resulted in losses of activity. Although the basis of *B. subtilis* urease inactivation is incompletely defined, high salt concentrations (0.5 to 1.5 M KCl) caused UreA dissociation from the heterotrimeric enzyme, with UreBC precipitating out of the solution. Urease in cell extracts was highly enriched (~85% homogeneous according to the integrated band intensities [Kodak 1D Scientific Imaging Systems]) by Sephacryl S-300 chromatography with 20 mM Tris-Cl, 150 mM NaCl, and 1 mM EDTA buffer, pH 7.4. By inductively coupled plasma emission analysis (Chemical Analysis Laboratory, University of Georgia), two independent preparations of *B. subtilis* urease contained 0.13 to 0.29 mol of Ni and 0.063 to 0.070 mol of Zn per mole of αβγ, with no significant levels of other metals. The Ni^{2+} content roughly correlates with the observed activity level, i.e., ~0.2 Ni/αβγ correlates to ~1% dinuclear center.

Direct comparison of recombinant expression of *B. subtilis ureABC* and *K. aerogenes ureABC*. The urease activity in *E. coli* cells expressing *B. subtilis ureABC* prompted us to reevaluate recombinant cells containing only *K. aerogenes ureABC*. Prior studies suggested that the *K. aerogenes* structural genes were ineffective in producing functional enzyme (24), but a very low level of activity would have been undetected. To directly compare the two systems, pURE93 and pKAU602 were constructed by using PCR and site-directed mutagenesis to contain *ureABC* of *B. subtilis* and *K. aerogenes* with the same pET42b expression vector and cloning strategy. The plasmids were transformed into *E. coli* C41(DE3) and the cells grown identically. *B. subtilis ureABC* was expressed at higher levels than *K. aerogenes ureABC* (Fig. 2), and in both cases, the UreA subunit was overproduced compared to the other subunits. Excess UreA synthesis may be due to the efficient ribosome binding site provided by the expression vector, whereas this was not observed with pURE91 or pKK17 (29), for which *ureA* expression uses the endogenous ribosome binding sites.

Urease activities were measured in extracts of the two *E. coli* transformants cultured with 7 mM $NiCl_2$ with or without IPTG (isopropyl-β-D-thiogalactopyranoside). Induced cell extracts containing *B. subtilis* and *K. aerogenes* UreABC exhibited ~9 U/mg and ~0.4 U/mg (Table 1), in approximate correspondence to the amount of UreC observed (Fig. 2). Although the level of activity found in cells harboring pKAU602 was low compared to that for cells containing pURE93, this result

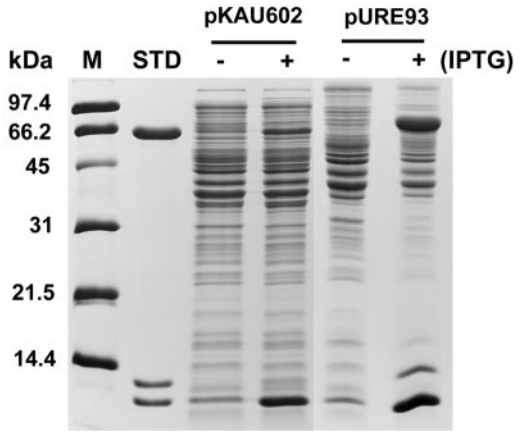

FIG. 2. Direct comparison of the levels of expression of *K. aerogenes ureABC* and *B. subtilis ureABC* from pET-42b derived vectors. Cultures of *E. coli* C41(DE3) cells carrying pKAU602 or pURE93 were induced with 0.5 mM IPTG, and the cell extracts were analyzed by denaturing polyacrylamide gel. Lanes: M, molecular weight markers; STD, *K. aerogenes* urease standard; pKAU602 − and + (IPTG), cell extracts containing *K. aerogenes* UreABC; pURE93 − and + (IPTG), cell extracts containing *B. subtilis* UreABC.

overturns prior dogma about *ureDEFG*-encoded accessory proteins being required for urease activation.

To investigate the in vitro activation properties of *K. aerogenes* and *B. subtilis* UreABC, activation was performed with cell extracts from transformants cultured without supplemental Ni^{2+} (Table 1). For pKAU602, no activity was detected prior to activation, consistent with the ability of TB medium to sequester trace levels of the required metal ion, while in vitro activation yielded ~0.8 U/mg. For pURE93, the level of trace activity increased to 1.3 U/mg after activation.

Coexpression studies with urease accessory genes. We tested whether coexpression of *B. subtilis ureABC* with *ureEFGD* from *K. aerogenes* or *Bacillus pasteurii* would affect activity. Plasmid pACT-ABCdel was constructed by deleting *ureABC* from the *K. aerogenes* urease operon in pACT-KKWT (25) by using the QuickChange mutagenesis kit (Stratagene). pACT-BpEFGD (carrying *B. pasteurii ureEFGD*) was generated by PCR-based cloning by using pBU11 (17) as a template and pACT3 (13) as a vector. pACT, pACT-ABCdel, and pACT-BpEFGD were cotransformed with pURE93 into the *E. coli* host, and pACT and pACT-ABCdel were cotransformed with pKAU602. Gene expression from each of the vectors was visible (data not shown). While the compatible vectors containing the cognate *K. aerogenes* genes yielded high levels of urease activity, coexpression of pURE93 and pACT-ABCdel did not enhance the activity over that for pURE93 alone (Table 2). Rather, the observed level of activity decreased in the cotransformant compared to pURE93 alone, perhaps due to reduced expression efficiency for two plasmids versus one plasmid in the same host. This decrease in urease activity also occurred in the pACT3 control. Similarly, the *B. pasteurii* accessory proteins failed to enhance activity from the *B. subtilis* structural genes (Table 2). We conclude that the *B. subtilis* urease subunits do not interact with heterologous urease accessory proteins from *K. aerogenes* or *B. pasteurii*. It remains possible that endogenous *E. coli* components facilitate synthesis of active urease expressed from *B. subtilis* or *K. aerogenes ureABC*. One possibility for a facilitator protein is SlyD, known to assist in activation of Ni-containing hydrogenase (40). Countering the participation of SlyD in these constructs are studies showing no effect when the intact *K. aerogenes* urease gene cluster or that from which *ureE* was deleted was transformed into *slyD* versus wild-type *E. coli* cells (6). To summarize, trace levels of recombinant *B. subtilis* urease are activated in *E. coli* without the participation of known urease accessory genes.

Evidence for novel accessory proteins in *B. subtilis*. Although *B. subtilis* lacks homologues to the established urease accessory genes, two lines of evidence support the existence of nonhomologous accessory gene(s) located at a locus (loci) separated from the subunit genes. First, the activity in *B. subtilis* is comparable to that in the recombinant *E. coli* cells, despite the vast overproduction of urease protein in the latter cultures. This result suggests an increased efficiency of activation in *B. subtilis* that could arise from increased intracellular Ni^{2+} or bicarbonate concentrations, folding issues in the heterologous host, or a novel accessory gene(s). Second, *B. subtilis* cells overexpressing recombinant *B. subtilis ureABC* lack enhanced urease activity. These cells are expected to contain Ni^{2+} and bicarbonate concentrations equivalent to those of the wild-type *B. subtilis* cells, and the folding machinery acts on homologous proteins, yet a much lower proportion of urease is activated. An unidentified accessory protein acting stoichiometrically could account for these results. Further studies are required to examine whether *B. subtilis* possesses one or more unidentified accessory genes.

TABLE 1. Urease activity in recombinant *E. coli* C41(DE3) cell extracts containing the indicated plasmids grown with 7 mM Ni^{2+} or grown without supplemental Ni^{2+} and subjected to activation conditions

Extracts	Sp act (μmol of urea/min/mg)	
	Without IPTG	With IPTG
Extracts of cells grown with Ni		
pKAU602	0.0463	0.438
pURE93	0.756	9.11
Extracts of induced cells without Ni	Without activation	With activation
pKAU602	ND[a]	0.838
pURE93	0.144	1.344

[a] ND, not detected.

TABLE 2. Urease activity from *E. coli* cotransformants grown in medium containing 5 mM $NiCl_2$

Plasmid	Sp act (μmol of urea/min/mg)	
	Without IPTG	With IPTG
pKAU602	0.0463	0.4129
pKAU602 + pACT3	0.0765	0.114
pKAU602 + pACT-ABCdel	12.684	70.377
pURE93	0.7556	9.114
pURE93 + pACT3	0.298	1.59
pURE93 + pACT-ABCdel	0.987	2.5
pURE93 + pACT-BpEFGD	0.066	0.154

Other urealytic systems lacking accessory proteins. Over 200 microbial genomes have been sequenced, and approximately 20% contain homologues to *ureC*. Additional targeted sequence information is available for urease gene clusters of numerous microorganisms. For most bacteria, the structural genes cluster with the accessory genes (with *ureD* sometimes referred to as *ureH*) in various arrangements (21, 22). Urease genes of selected microorganisms are interrupted by intervening sequences, such as the six open reading frames within *ureABCDEFG* of *Agrobacterium tumefaciens*. Similarly, *ureDABC* and *ureEFG* of *Pseudomonas aeruginosa* PAO1 and *Pseudomonas syringae* pv. tomato strains are separated by more than 15,000 bp (with additional open reading frames between *ureA* and *ureB*). *Synechocystis* sp. strain PCC 6803 and *Thermosynechococcus elongatus* BP-1 have urease genes dispersed throughout their genomes. Of greater relevance to the *B. subtilis* system are cases in which one or more urease accessory genes are missing. The urease gene cluster of the *Mycobacterium tuberculosis* Erdman strain contains only *ureABCFG*, yet it synthesizes active urease (10); however, the missing genes may be elsewhere on the chromosome. In contrast, the genomes of *M. tuberculosis* CDC1551, *M. tuberculosis* H37Rv, and *Mycobacterium bovis* AF2122/97, "*Candidatus* Blochmannia floridanus," *Streptomyces avermitilis* MA-4680, *Streptomyces coelicolor* A3 (2), *Bradyrhizobium japonicum*, *Rhodopseudomonas palustris* CGA009, and *Nocardia farcinica* lack homologues of *ureE*. Still, *B. subtilis* is the only urealytic organism that lacks all known accessory genes.

Activation of nonurease dinuclear hydrolases. Phosphotriesterase (5), dihydro-orotase (34), isoaspartyl dipeptidase (33), and three different hydantoinases (1, 2, 9) all contain active sites closely resembling that of urease (with a carbamylated lysine bridging two metal ions, typically zinc), yet no genetic or biochemical evidence implicates accessory proteins for their biosynthesis. We suggest that *B. subtilis* urease provides a link between activation of these enzymes and that of the typical urease systems. Urease can be activated without accessory proteins, but the efficiency is very low; accessory proteins greatly enhance the efficiency by a still poorly understood process coupled with GTP hydrolysis. Of related interest, the activation of Ni-containing hydrogenases, carbon monoxide dehydrogenase, and acetyl-coenzyme A decarbonylase-synthase also requires accessory genes for efficient metallocenter assembly (23).

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