Regulation of Gene Expression and Cellular Localization of Cloned *Klebsiella aerogenes* (*K. pneumoniae*) Urease

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The genes for *Klebsiella aerogenes* (*K. pneumoniae*) urease were cloned and the protein was overexpressed (up to 18% of total protein consisted of this enzyme) in several hosts. The small size of the DNA encoding urease (3.5 kb), the restriction map, and the regulation of enzyme expression directed by the recombinant plasmid are distinct from other cloned ureases. Nickel concentration did not affect urease gene expression, as demonstrated by the high levels of apoenzyme measured in cells grown in nickel-free media. However, nickel was required for urease activity. The overproducing recombinant strain was used for immunogold electron microscopic localization studies to demonstrate that urease is a cytoplasmic enzyme.

INTRODUCTION

Nickel-containing ureases (EC 3.5.1.5), which hydrolyse urea to ammonia and carbon dioxide, play an important role in nitrogen metabolism of plants and micro-organisms (Hausinger, 1987). One of the best-studied bacterial ureases is that from *Klebsiella aerogenes* [currently *Klebsiella pneumoniae* (Ørskov, 1974)]; its regulation has been characterized (Friedrich & Magasanik, 1977; Magasanik, 1982) and the three-subunit enzyme has been purified and shown to contain four nickel ions per native molecule (Todd & Hausinger, 1987). Our efforts are geared toward elucidating the structure and function of the *K. aerogenes* urease nickel centre by using chemical, biophysical and spectroscopic approaches which require large amounts of enzyme. Typical yields of urease are only 0.1 mg per litre of culture. Therefore, we sought to clone the *K. aerogenes* urease genes and to define conditions needed to optimize enzyme overexpression. In addition, the effect of nickel concentration on urease activity and expression was characterized and the cellular location of recombinant urease was defined.

METHODS

Gene cloning. *K. aerogenes* CG253 was obtained from Boris Magasanik and Alex Ninfa (Massachusetts Institute of Technology). Chromosomal DNA of *K. aerogenes* was isolated (Miller, 1972) and partially digested with *Sau*3A to yield fragments approximately 40 kbp in size. After phenol extraction and ethanol precipitation, the digestion mixture was ligated to *Bam*HI-cleaved, phosphatase-treated, cosmid vector pWH4 (Herrera *et al.*, 1984) and the resulting DNA was packaged into λ phage by using an *in vitro* packaging system (Boehringer Mannheim) according to the manufacturer's instructions. The phages were used to transfect *Escherichia coli* strain VCS257 (Stratagene) and kanamycin-resistant (50 μ g ml⁻¹) colonies were screened on urease indicator plates, which consisted of ammonia-free M9 minimal agar (Maniatis *et al.*, 1982) adjusted to pH 6.8 and supplemented with 10% (v/v) LB medium, 20 mM-urea, 20 μ g phenol red ml⁻¹ and 1 ml trace mineral solution l⁻¹ (Smith *et al.*, 1980). One of 102 colonies tested positive, as shown by the development of a red halo after 24 h. The cosmid which encoded urease (designated pKAU1) was isolated (Kado & Liu, 1981) and transformed into *E. coli* DH1 (Hanahan, 1983); this plasmid conferred both kanamycin-resistance and urease-positive phenotypes. Purified cosmid pKAU1 was digested with *Bam*HI, and the fragments were ligated into *Bam*HI-cleaved vector pBR328 (Soberon *et al.*, 1980).

One ampicillin-resistant, tetracycline-sensitive transformant was positive on urease indicator plates, and was found to contain a 10 kb insert in a plasmid derivative of pBR328 which was termed pKAU2687. Restriction fragments of the *K. aerogenes* insert in pKAU2687 were isolated (Dretzen *et al.*, 1981), subcloned into vector pUC8 (Vieira & Messing, 1982), transformed into *E. coli* JM101 (Messing *et al.*, 1981) or JM107 (Yanisch-Perron *et al.*, 1985), and screened on urease indicator plates.

Assays. Culture samples of cells grown in various media (0.5 ml) were centrifuged for 2 min in an Eppendorf centrifuge at 4 °C, washed twice with 10 mM-potassium phosphate, 1 mM-EDTA, 1 mM-2-mercaptoethanol buffer (pH 7.5), and resuspended in the same buffer plus 0.5 mM-phenylmethanesulphonyl fluoride. Cells were disrupted with a Fisher Sonic Dismembrator (micro probe) using three 20 s bursts at 30% power. Crude cell extracts were then centrifuged for 15 min and the supernatant solutions were assayed for urease activity by converting released ammonia to indophenol, which was quantified spectrophotometrically (Todd & Hausinger, 1987). Protein was assayed by the Lowry method, with bovine serum albumin as the standard.

Nickel-dependence studies. The effect of nickel concentration on the expression of recombinant urease protein and enzyme activity was determined by growing cultures to late exponential phase in ammonia-free MOPS minimal medium (Neidhardt *et al.*, 1974) containing defined nickel levels. Glutamine (10 mM) was used as the sole nitrogen source in order to derepress urease (see below); however, under these conditions urease activity was not required for microbial growth.

Immunological methods. Antibodies that recognized urease were generated in a New Zealand rabbit after injection with homogeneous enzyme, and the IgG fraction was purified from serum (McKinney & Parkinson, 1987). For immunoblot analyses, samples were denatured, electrophoresed on an SDS 10–15% acrylamide gradient gel (Laemmli, 1970), and blotted onto nitrocellulose. The blot was probed with anti-*K. aerogenes* urease antibodies and developed by using anti-rabbit IgG-alkaline phosphatase conjugates (Blake *et al.*, 1984).

Immunogold electron microscopy. Wild-type *K. aerogenes* and *K. aerogenes*(pKAU19) (see below) were grown to stationary phase in ammonia-free MOPS medium supplemented with 10 mM-arginine plus 100 µM-NiCl₂. After centrifugation, the cells were washed once in 10 mM-potassium phosphate, 1 mM-EDTA (pH 7), and fixed in 0.1 M-potassium phosphate (pH 7.2) containing 1% (v/v) glutaraldehyde for 30–60 min at room temperature. The fixed cells were resuspended in 1% (w/v) Nobel agar, dehydrated in ethanol, and embedded in Lowicryl K4M (Armbruster *et al.*, 1982). Polymerization was carried out for 2 d at room or cold-room temperatures under UV irradiation. Thin sections were cut by using an LKB Ultratome III microtome, and placed on Butvar B-98-coated nickel grids. Sections were floated first on a drop of TBST (Tris-buffered saline, pH 7.4, with 0.05% Tween 20) for 5 min and transferred to 1% (w/v) bovine serum albumin in TBST for 15 min in order to block non-specific sites. The samples were transferred to the anti-urease IgG (35 µg ml⁻¹) in TBST for 1 h, washed three times for 5–15 min each in TBST, and floated on gold particles that were attached to anti-rabbit IgG (15 nm, Jansen) for 1 h (Bendayan, 1984). After washing in TBST and H₂O, the samples were stained with uranyl acetate and lead citrate. Sections were observed with a Philips CM-10 electron microscope.

RESULTS AND DISCUSSION

Gene cloning

The *K. aerogenes* urease genes were localized to a 10 kbp DNA fragment which possesses the restriction map shown in Fig. 1. This region included two *Bam*HI fragments of 7.0 and 3.0 kbp, indicating that incomplete digestion had occurred in the subcloning of pKAU2687. When cloned individually, neither fragment conferred urease activity, indicating that sequences spanning the internal *Bam*HI site are required. A *Pvu*II fragment (6.2 kbp) overlapped this *Bam*HI site, yet no urease activity was detected in transformants containing this subcloned fragment (pKAU13). Partial *Sau*3A digestion of pKAU15 was used to generate clones containing 5.7 kbp (pKAU17) and 3.5 kbp (pKAU23) fragments, both of which conferred urease activity. The 3.5 kbp insert is the smallest reported fragment from any micro-organism which is sufficient to encode active urease.

Urease genes have been cloned from *Bacillus pasteurii* (Kim & Spizizen, 1985), a urease-positive *E. coli* (Collins & Falkow, 1988), *Proteus mirabilis* (Jones & Mobley, 1988; Walz *et al.*, 1988), and *Providencia stuartii* (Mobley *et al.*, 1986; Mulrooney *et al.*, 1988). The restriction maps of these clones all differ significantly from that of the *K. aerogenes* urease gene fragment. *Klebsiella pneumoniae* urease genes also have been cloned by Gerlach *et al.* (1988); however, the mechanism of gene regulation and properties of the recombinant enzyme were not studied. The restriction pattern, and the associated polypeptide sizes for the *K. pneumoniae* urease genes studied by Gerlach *et al.* (1988) differ from that of the *K. aerogenes* DNA cloned in the present study.

Fig. 1. Restriction map and summary of cloned *K. aerogenes* urease gene fragments. A restriction map is presented of a 10 kb DNA fragment containing the *K. aerogenes* urease genes. *Hind*III, *Eco*RI and *Xba*I did not cleave this fragment. Subclones of the *K. aerogenes* DNA fragment were generated and tested for the presence (+) or absence (—) of urease activity based on results from indicator plates.

Urease regulation

To increase their expression, the cloned urease genes were transformed into three hosts and the enzyme levels were monitored under varied growth conditions. The 5.7 kbp fragment was transferred from pUC8 to pBR328, which had more desirable antibiotic markers: pKAU17 was digested with *Eco*RI and *Hind*III, end-filled with polymerase 1 Klenow fragment, and ligated into *Sma*I-digested pBR328 to yield pKAU19. This plasmid was transformed into *E. coli* DH1 and *K. aerogenes* CG253 (Hanahan, 1983) or into *Salmonella typhimurium* LT-2 (MacLachlan & Sanderson, 1985). Since pKAU19 carried sequences homologous to the *K. aerogenes* host (which could lead to plasmid loss due to homologous recombination), colonies obtained directly from the transformation were used to inoculate starter cultures for overnight incubation. Specific activities were determined for cells of these strains and wild-type *K. aerogenes* grown to early exponential and stationary phase in different media that contained individual nitrogen sources; typical values are shown in Table 1. For comparison, pure *K. aerogenes* urease has a specific activity of 2200 $\mu\text{mol urea min}^{-1} \text{mg}^{-1}$ (Todd & Hausinger, 1987).

Among the hosts tested, urease activity was regulated by nitrogen repression (Magasanik, 1982) as originally reported in the wild-type microbe (Friedrich & Magasanik, 1977). Low enzyme levels were observed during growth in nitrogen-rich medium (LB, or MOPS + CA + N, Table 1), whereas nitrogen limitation (MOPS + N, Gln, or Arg) led to a derepression of urease activity. Enzyme levels exceeded 7% of the soluble protein in stationary-phase cultures of strains containing the cloned urease genes, and accounted for 18% in the case of *S. typhimurium* grown in MOPS + Arg calculated from the data in Table 1. However, the highest activity per ml of culture was obtained for *K. aerogenes*(pKAU19). Regulation of other recombinant ureases has only been reported for urea-inducible, nitrogen-repressible *Providencia stuartii* (Mulrooney *et al.*, 1988) and urea-inducible *Proteus mirabilis* (Jones & Mobley, 1988; Walz *et al.*, 1988).

Table 1. *Levels of urease activity in strains containing the cloned urease genes of K. aerogenes*

Time of harvest* and culture medium†	Culture specific activity [$\mu\text{mol urea min}^{-1}$ (mg protein) $^{-1}$]			
	<i>K. aerogenes</i> wild-type	<i>K. aerogenes</i> (pKAU19)	<i>S. typhimurium</i> (pKAU19)	<i>E. coli</i> (pKAU19)
Exponential growth phase				
LB	0.0	6.1	2.5	1.4
MOPS + CA + N	0.1	6.4	11.2	9.1
MOPS + N	0.3	49	111	107
MOPS + Gln	0.4	34	48.3	109
MOPS + Arg	3.3	122	29.5	165
Stationary growth phase				
MOPS + Gln	1.1	263	219	119
MOPS + Arg	1.7	240	403	176

* Exponential phase cultures were harvested at $\text{OD}_{600} = 1$.

† The media were either LB (Luria-Bertani medium; Maniatis *et al.*, 1982) or MOPS minimal medium (Neidhardt *et al.*, 1974). For the latter, the nitrogen source was altered to include 0.5% Casamino acids (CA), 10 mM-NH₄Cl (N), 10 mM-glutamine (Gln) or 10 mM-arginine (Arg). All cultures contained 100 μM -NiSO₄ and plasmid-bearing cultures contained 30 μg chloramphenicol ml⁻¹.

Urease activity in *E. coli* JM107(pKAU23) was only 1% of that observed for pKAU19; nevertheless the same pattern of regulation was demonstrated. In addition, immunoblot analysis of this clone demonstrated that all three urease polypeptides were present at levels consistent with the activity (data not shown), i.e. the reduced activity of the 3.5 kbp clone is not due to production of low-activity protein. The larger DNA fragment may possess ancillary genes which enhance the levels of urease expression. In this regard, minicell analysis has demonstrated the presence of multiple, non-urease genes in the urease operon of other species (Gerlach *et al.*, 1988; Jones & Mobley, 1988; Mulrooney *et al.*, 1988; Walz *et al.*, 1988).

Characterization of the urease made by strains containing pKAU19

Crude cell extracts of *E. coli* DH1(pKAU19) and *S. typhimurium*(pKAU19) were examined by using immunoblot analysis (Fig. 2). Three urease subunits of the expected size were shown to be expressed in each case. These results demonstrate that urease-processing or nickel-insertion activities, if required, are encoded either on the 5.7 kbp pKAU19 fragment or in the heterologous hosts. Furthermore, enzyme purified from *K. aerogenes*(pKAU19) was demonstrated to be identical to wild-type enzyme in subunit composition, nickel content, specific activity, and inhibitor sensitivity (data not shown). Routine purification of urease is now carried out from stationary-phase cultures of *K. aerogenes*(pKAU19), resulting in 100- to 200-fold increases in yield over wild-type levels.

Effects of nickel concentration on urease gene expression and urease activity

Derepressed, stationary-phase *K. aerogenes*(pKAU19) cultures grown in media of different nickel concentrations yielded identical intensities of anti-urease antibody cross-reactive material (Fig. 3). Hence, nickel does not affect expression of urease in strains with this plasmid. The urease activities, however, were greatly affected by nickel concentration, and 100–200 μM -nickel was required for maximum activity (Fig. 3). The high requirement may reflect the ability of medium components to bind nickel. The production of inactive urease apoenzyme by this enteric bacterium is similar to the case of soybean, where apoenzyme was shown to be synthesized in the absence of nickel (Winkler *et al.*, 1983). Furthermore, the algae *Phaeodactylum tricornutum* and *Tetraselmis subcordiformis* (Rees & Bekheet, 1982), the cyanobacterium *Anabaena cylindrica* (Mackerras & Smith, 1986), and the purple sulphur bacterium *Thiocapsa roseopersicina* (Bast, 1988) have also been suggested to synthesize urease apoenzyme in the absence of nickel. The microbial studies were based on reconstitution of urease activity for cells grown under nickel-free conditions when nickel was added, even in the

Fig. 2

Fig. 3

Fig. 2. Immunoblot analysis of recombinant urease expressed in *E. coli* DH1 and *S. typhimurium*. Samples of crude extracts of *E. coli* DH1(pKAU19) (lane 1) and *S. typhimurium*(pKAU19) (lane 2) containing 0.1 units (50 ng) of urease were analysed. A standard of purified *K. aerogenes* urease (lane 3) and M_r standards (lane 4) were also run and stained for total protein with Amido black. The migration position for M_r markers (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme; Bio-Rad) are indicated to the right of the figure.

Fig. 3. Effect of nickel concentration on recombinant urease expression and activity. (a) Urease specific activity was determined for *K. aerogenes*(pKAU19) after growth in media containing the indicated nickel concentrations. (b) The level of urease protein expression was quantified for each sample by immunoblot staining. Lanes 1 and 2 are Amido-black-stained M_r standards and purified urease, respectively. Lanes 3–9 represent the samples (2 μg protein) obtained from cultures containing 0, 12.5, 25, 50, 100, 200, and 400 μM-nickel, respectively.

presence of protein-synthesis inhibitors. In contrast to the lack of nickel-dependent regulation of ureases, nickel-containing hydrogenases from *Bradyrhizobium japonicum* and *Alcaligenes latus* have been shown to exhibit nickel-dependent expression (Stults *et al.*, 1986; Doyle & Arp, 1988).

Immunogold localization

The urease in cells of *K. aerogenes* containing pKAU19 is a cytoplasmic enzyme as shown by immunogold electron microscopy localization (Fig. 4). This result is consistent with the observed enzyme behaviour during purification. Urease from wild-type *K. aerogenes* behaved identically to recombinant enzyme during isolation, which is consistent with the reported cytoplasmic location from cell fractionation studies (Friedrich & Magasanik, 1977). Wild-type cells which contain 1–5 μM-urease (calculated as in Kellenberger *et al.*, 1987), were insufficiently labelled by the immunogold technique to allow localization, as expected from the findings of Kellenberger *et al.* (1987), who state that a cytoplasmic protein cannot be detected by this method at concentrations less than 10–100 μM.

Fig. 4. Immunogold localization of recombinant *K. aerogenes* urease. (a) Thin sections of *K. aerogenes*(pKAU19) expressing recombinant urease ($247 \mu\text{mol}$ urea hydrolysed $\text{min}^{-1} \text{mg}^{-1}$) were reacted with anti-urease antibodies and labelled with anti-rabbit IgG-gold particles. Urease was localized to the cytoplasmic portion of the cell. (b) Similar experiments were carried out with wild-type *K. aerogenes* ($2.0 \mu\text{mol}$ urea hydrolysed $\text{min}^{-1} \text{mg}^{-1}$). Bar, $0.2 \mu\text{m}$.

Our results contrast with two previous electron microscopic localization studies involving urease from a *Staphylococcus* species (McLean *et al.*, 1985) and from *Proteus mirabilis* (McLean *et al.*, 1986). The earlier workers utilized tetraphenylboron, a compound which reacts with ammonia to form a precipitate; ammonia was exchanged for electron-dense silver ions to allow visualization by electron microscopy after thin sectioning. The precipitated metal was observed on the membrane of the Gram-positive *Staphylococcus* sp. (McLean *et al.*, 1985) or in the periplasmic space and on the outer membrane of the Gram-negative *P. mirabilis* (McLean *et al.*, 1986). The discrepancy between these earlier studies and our findings may be due to the inability of tetraphenylboron to cross the cytoplasmic membrane, thus it could only react with external ammonia.

Note added in proof: recent analyses of *E. coli*(pKAU23) have not exhibited any urease activity, in contrast to the low levels described above. Thus, the 3.5 kbp DNA fragment may not contain all of the *K. aerogenes* urease genes.

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