JOURNAL OF BACTERIOLOGY, Jan. 1991, p. 472–478
0021-9193/91/020472-07$02.00/0

Vol. 173, No. 2

Choline Oxidase, a Catabolic Enzyme in *Arthrobacter pascens*, Facilitates Adaptation to Osmotic Stress in *Escherichia coli*†

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Received 30 July 1990/Accepted 24 October 1990

Choline oxidase (EC 1.1.3.17) is a bifunctional enzyme that is capable of catalyzing glycine betaine biosynthesis from choline via betaine aldehyde. A gene (*cox*) encoding this enzyme in the gram-positive soil bacterium *Arthrobacter pascens* was isolated and characterized. This gene is contained within a 1.9-kb fragment that encodes a polypeptide of approximately 66 kDa. Transfer of this gene to an *Escherichia coli* mutant that is defective in betaine biosynthesis resulted in an osmotolerant phenotype. This phenotype was associated with the ability of the host to synthesize and assemble an enzymatically active choline oxidase that could catalyze biosynthesis of glycine betaine from an exogenous supply of choline. Although glycine betaine functions as an osmolyte in several different organisms, it was not found to have this role in *A. pascens*. Instead, both choline and glycine betaine were utilized as carbon sources. In *A. pascens* synthesis and activity of choline oxidase were modulated by carbon sources and were susceptible to catabolite repression. Thus, *cox*, a gene concerned with carbon utilization in *A. pascens*, was found to play a role in adaptation to an environmental stress in a heterologous organism. In addition to providing a possible means of manipulating osmotolerance in other organisms, the *cox* gene offers a model system for the study of choline oxidation, an important metabolic process in both procaryotes and eucaryotes.

Adaptation to osmotic stress is fundamental to survival and growth of organisms. As such, it exemplifies the intimate relationship between an organism and its environment. Of the various means evolved to overcome osmotic stress, accumulation of solutes to provide osmotic balance with the environment is a commonly used strategy in a diverse set of organisms (14, 17, 23, 51, 53, 54). Inorganic ions play a principal role in this regard, but high intracellular concentrations can be detrimental to cellular functions (54). Many organisms appear to overcome this problem by accumulating one or more organic compounds, such as polyols, amino acids, and quaternary ammonium compounds, that can act as compatible solutes (54). These compounds may also directly protect enzymes and other cellular components from high salt concentrations (36).

The osmoprotective role of the quaternary ammonium compound glycine betaine (*N*,*N*,*N*-trimethylglycine; betaine) is evident in a number of diverse microbial systems, including enteric bacteria (1), soil bacteria (45), halophilic bacteria (15), cyanobacteria (27), and methanogenic archaebacteria (39). Accumulation of betaine as an adaptive strategy has also been demonstrated in plants (51, 53). It has been suggested that betaine functions as an osmolyte in the mammalian renal system as well (3). Besides this physiological role as an osmoprotectant, betaine also functions in general metabolism where methyl groups derived from it are incorporated into alkaloids in plants (9), into methionine in mammals (43) and microorganisms (52), and into cobalamin (vitamin B_{12}) in microorganisms (52). Furthermore, betaine (and its precursor, choline) can be used as a carbon and nitrogen source by some microorganisms (21). Methyl groups liberated by betaine catabolism in these microbial systems may be assimilated or oxidized to carbon dioxide via one-carbon metabolism pathways (24, 25).

Biosynthesis of betaine results from oxidation of choline via a two-step reaction with betaine aldehyde as the intermediate. This series of reactions may be catalyzed by three different enzymatic systems. In microorganisms and mammals a membrane-bound choline dehydrogenase (EC 1.1.99.1) is employed in conjunction with a soluble betaine aldehyde dehydrogenase (EC 1.2.1.8) (18, 22, 31, 32, 40). Plants utilize a soluble choline monooxygenase in combination with betaine aldehyde dehydrogenase (7, 34). A third choline oxidation system, as yet found only in microorganisms, involves a soluble choline oxidase that is capable of catalyzing both reactions in vitro (20, 33, 49).

The involvement of choline oxidase with betaine biosynthesis suggests a possible role in osmotolerance. To investigate this prospect and to evaluate a possible means for genetic engineering of enhanced betaine synthesis and osmotolerance in biological systems, we have cloned and characterized an *Arthrobacter pascens* gene (*cox*) coding for a choline oxidase. Although *cox* was found to be involved only in carbon utilization in this gram-positive organism, its expression in an *Escherichia coli* strain resulted in an osmotolerant phenotype.

MATERIALS AND METHODS

Bacterial strains, vectors, and growth conditions. The following *Arthrobacter* and *Alcaligenes* strains were obtained from the American Type Culture Collection: *Arthrobacter aurescens* ATCC 13344; *Arthrobacter globiformis* ATCC 4336; *A. pascens* ATCC 13346; *Alcaligenes* sp. strains ATCC 11451, ATCC 11884, and ATCC 27066; *Alcaligenes faecalis* ATCC 8750; *Alcaligenes hydrogenophilus* ATCC 33178; *Alcaligenes marshalli* ATCC 21030. *E. coli* strains include DH5α [Bethesda Research Laboratories; F^- φ80

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† NRCC publication no. 32463.

d*lacZ*ΔM15 *endA1 recA1 hsdR17 supE44 thi-1* λ⁻*gyrA96* Δ(*argF-lac*)*U169*] and MC4100 [11; F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*]. Cloning vectors used include λZAPII (42), pBluescript SK− (42), pBR322 (4), pHG329 (46), and pKK223-3 (6).

Arthrobacter and *Alcaligenes* strains were cultured by using growth conditions recommended by the ATCC or in the medium of Ikuta et al. (20; referred to as Ikuta medium). The micronutrient components of a synthetic medium described by Repaske and Repaske (37; referred to as Repaske medium) were used in some experiments. For visual estimation of osmotolerance, overnight nutrient broth cultures of *A. pascens* were washed with and suspended in 1 volume of Repaske medium. Then 40 μl of the cell suspension was streaked for single colonies in a consistent manner for all plates. Repaske medium containing 0.2% (wt/vol) glucose, choline, or betaine or containing 0.2% (vol/vol) glycerol was used for the controls. NaCl (0.6 M) was added to provide a minimal inhibitory osmotic stress as determined empirically in prior experiments; lower concentrations promoted higher background growth, and ≥1.0 M NaCl abolished growth completely. All cultures of *A. pascens* were incubated at 30°C.

E. coli strains were cultured at 37°C in LB medium (2) or, for osmotolerance experiments, in M63 medium (28) supplemented with 0.5% (vol/vol) glycerol, and 1 mM choline, betaine aldehyde, or betaine. Osmotic stress was provided by the addition of 0.65 M NaCl. Aliquots from overnight cultures in M63 medium were streaked for single colonies on the various salt media. All media were solidified with 1.5% (wt/vol) agar where required.

Immunological and biochemical screening for choline oxidase. *Alcaligenes* and *Arthrobacter* strains were screened for the presence of choline oxidase by immunoblot analysis (8) with choline oxidase antibodies and by biochemical assay. Polyclonal antibodies were raised in rabbits against purified choline oxidase from *A. globiformis* (Boehringer Mannheim) and *Alcaligenes* sp. (Sigma). The specificity of the antiserum was evaluated by comparison of immunoblot analyses (8) with the antiserum and affinity-purified antibodies (44). It was further confirmed by immunoprecipitation experiments. Immunoprecipitation was done by incubating aliquots of the antiserum with choline oxidase from *A. globiformis* in 10 mM Tris hydrochloride (pH 8.0)–150 mM NaCl at 37°C for 90 min. The reaction material was then centrifuged at 1,000 × *g* for 10 min at room temperature. The supernatant was assayed for choline oxidase activity as described by Ikuta et al. (20).

For immunoblot and enzyme assays of the strains, cells were cultured in Ikuta medium, harvested during exponential growth, and lysed with a French press (7,000 lb/in²; American Instrument Co., Inc.) or by sonication (5 to 10 min at 40 W; Braun-Sonic 2000 sonicator). The protein content of cell lysates was determined by the method of Bradford (5). One unit of choline oxidase corresponds to 1 μmol of H_2O_2 produced per min (20).

Construction of the genomic library of *A. pascens* and related methods. *A. pascens* DNA was prepared by suspending the cells from overnight cultures in 10 mM Tris hydrochloride (pH 8.0)–25 mM EDTA–150 mM NaCl. Lysozyme was added to a concentration of 20 mg/ml, and the cells were frozen and thawed five times before the suspension was incubated at 37°C for 30 to 60 min. The lysate was then extracted with an equivalent volume of TE (100 mM Tris hydrochloride [pH 8.0], 10 mM EDTA)-buffered phenol and then extracted with chloroform-isoamyl alcohol (24:1). DNA was precipitated with 2 volumes ethanol, suspended in 0.1× TE, and partially digested with *Sau*3AI (New England BioLabs). The DNA was size fractionated by sucrose density gradient centrifugation (2), and the fractions containing 4- to 9-kb fragments were pooled. *Sau*3AI ends were partially filled in with dGTP and dATP by using the Klenow fragment of DNA polymerase (Pharmacia LKB). λZAPII DNA with ligated cohesive ends was cut with *Xho*I and partially filled in with dCTP and dTTP. The lambda arms and insert DNA were ligated overnight at 7°C (T4 DNA ligase; New England BioLabs). Packaging in vitro was done according to the instructions supplied with the Gigapack Gold kit of Stratagene. The gene library was amplified once on agar overlay plates with *E. coli* DH5α as the host.

Immunoscreening of the library with choline oxidase antibodies was as described by Short et al. (42). Isolation of a choline oxidase-proficient clone involved the following: (i) choline oxidase assay of MC4100 lysogens constructed with phages from pooled immunopositive plaques (10 plaques per pool, 10 pools in total); (ii) assays of individual lysogens constructed with phages from a Cox^+ pool; (iii) confirmation by immunoblots of the Cox^+ lysogen; and (iv) excision of recombinant plasmids by the method of Short et al. (42), with the exception of using DH5 α/F′ (BRL) as the host.

Minipreparations of plasmid DNA, restriction digests, ligations, and agarose gel electrophoresis were done by standard protocols (2). Transformation of *E. coli* strains was as described by Morrison (30) or by the recently described method of Chung et al. (12).

RESULTS

***A. pascens* encodes a choline oxidase.** Nine *Arthrobacter* and *Alcaligenes* strains (see Materials and Methods) were screened for the presence of choline oxidase. Unconcentrated cell extracts were used to favor detection of strains with substantial choline oxidase activity. *A. pascens* ATCC 13346 contained the highest activity (12.5 U/mg of total protein). *A. aurescens* ATCC 13344 and *A. globiformis* ATCC 4336 also exhibited choline oxidase activity, whereas none of the *Alcaligenes* strains had the activity (<0.01 U/mg).

The biochemical screen was supplemented with an immunological assay with anti-choline oxidase antibodies raised against commercially available choline oxidase from *A. globiformis* and *Alcaligenes* sp. The two antisera were self-reactive with their respective antigens and also reciprocally cross-reactive (data not shown), indicating a serological relationship between choline oxidases from these two different bacterial sources. In addition, there were auxiliary cross-reactive polypeptides in both enzyme preparations revealed by their respective antisera (data not shown). However, the antibodies generated against the *A. globiformis* choline oxidase showed fewer and less intense auxiliary cross-reactive polypeptides; these additional polypeptides were found to be serologically related to choline oxidase by using affinity-purified antibodies (data not shown). The auxiliary polypeptides, therefore, likely represent degradation products of choline oxidase. The specificity of the antiserum for the enzyme was further examined by immunoprecipitation experiments, which demonstrated up to 75% reduction in choline oxidase activity after treatment with the antibody (data not shown). These results established the suitability of the *A. globiformis* choline oxidase antibodies for further experiments. Of the nine strains screened for the presence of choline oxidaselike polypep-

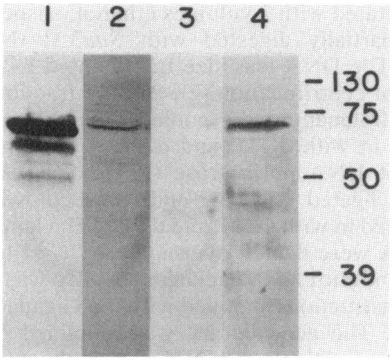

FIG. 1. Immunoblot analysis with choline oxidase antibodies. Lanes: 1, purified choline oxidase of *A. globiformis*; 2 through 4, crude extracts: 2, *A. pascens* ATCC 13346; 3, *E. coli* MC4100 lysogenic for λZapII vector; 4, *E. coli* MC4100 lysogenic for the recombinant phage, phage-8K. The molecular size standards (in kilodaltons) are indicated to the right.

tides, *A. pascens* ATCC 13346, *A. aurescens* ATCC 13344, and *A. globiformis* ATCC 4336 possessed a cross-reactive protein of 66 kDa (data not shown). Given this result and the higher choline oxidase activity of *A. pascens*, this strain was chosen for further studies.

Identification and characterization of a recombinant clone encoding the choline oxidase of *A. pascens*. Some 100 immunopositive plaques were isolated from the *A. pascens* genomic library after approximately 10^5 PFU were screened with the choline oxidase antibodies. Three of these recombinant phage conferred choline oxidase proficiency when used to lysogenize *E. coli* MC4100. One of these *E. coli* strains, KLR-1, containing a recombinant phage, phage-8K, was found to produce a cross-reactive polypeptide of the same molecular weight as that of the cross-reactive protein of *A. pascens* in immunoblot analysis with the choline oxidase antibodies (Fig. 1). Thus, given these results and the choline oxidase proficiency of KLR-1, phage-8K was presumed to contain a full-length clone of the *cox* gene of *A. pascens* encoding a choline oxidase.

The recombinant plasmid pKR1, excised from phage-8K, was digested with *Eco*RI, producing 4- and 1.8-kb fragments in addition to the vector. These two fragments were subcloned into the *Eco*RI site of pBluescript SK−. Placing the 4-kb *Eco*RI fragment in either orientation within pBluescript SK− (pKR11, pKR12) conferred choline oxidase proficiency to *E. coli* MC4100 (Fig. 2). This demonstrated that the 4-kb fragment contained a functional gene, and it further suggested that the *cox* promoter of *A. pascens* is active in *E. coli*. A physical map of the insert DNA is provided in Fig. 2. pKR11 with the *Eco*RV site adjacent to the *lac* promoter conferred approximately a 55-fold higher level of choline oxidase activity to *E. coli* MC4100 than did pKR12, which contains the insert in the opposite orientation (Fig. 2). This elevated expression of *cox*, presumably by the *lac* promoter from the vector, suggested that the orientation of the *cox* gene in this 4-kb fragment has the 5′ end of the gene toward the *Eco*RV site and the 3′ end toward the *Sca*I site (Fig. 2). To further characterize the configuration of the *cox* gene in the 4-kb clone, fragments of the insert DNA were deleted or subcloned into pBluescript SK− and pKK223-3 (Fig. 2). Deletion of either *Pvu*II fragment (coordinates: kb 0 to 1.2, pKR23; kb 1.2 to 4, pKR24) (Fig. 2) resulted in loss of choline oxidase activity. However, subcloning the large

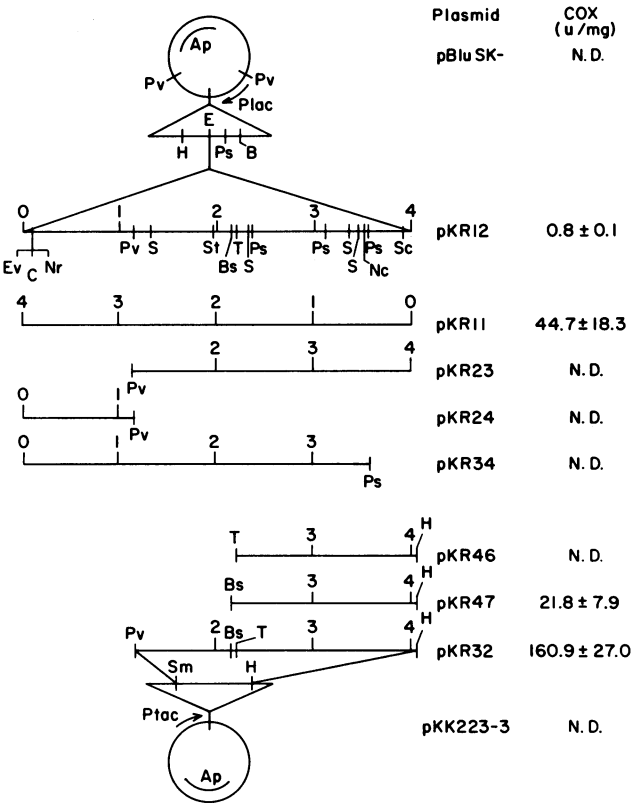

FIG. 2. Physical and genetic characterization of a *cox* clone. A 4-kb *Eco*RI fragment from the insert in phage-8K was subcloned into pBluescript SK− (pBlu SK− [42]) and pKK223-3 (6). The insert DNA was further characterized by deletion and subcloning as indicated. pKK223-3 is the vector in pKR32, pKR46, and pKR47; pBluescript SK− is the vector in other pKR plasmids. The coordinates are in kilobase pairs. Abbreviations: COX, choline oxidase activity in *E. coli* MC4100 derivatives grown in L broth as determined in three experiments; N.D., not detectable; Ap, ampicillin resistance; Plac, *lac* promoter; Ptac, *tac* promoter; B, *Bam*HI; Bs, *Bst*EII; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Nc, *Nco*I; Nr, *Nru*I; Ps, *Pst*I; Pv, *Pvu*II; S, *Sac*II; Sc, *Sca*I; Sm, *Sma*I; St, *Sfi*I; T, *Tth*111I.

*Pvu*II-*Hin*dIII fragment (coordinates: kb 1.2 to 4) of pKR11 into the *Sma*I-*Hin*dIII window of pKK223-3, downstream of the *tac* promoter, resulted in high levels of choline oxidase activity when this construct was present in *E. coli* MC4100 (pKR32; 160.9 U/mg). Since the cloning site of pKK223-3 does not include a translation initiation codon (6), the 2.8-kb insert must have its own initiation codon for expression. A further deletion of 900 bp was achieved by subcloning the *Bst*EII-*Hin*dIII fragment of pKR32 into the *Sma*I-*Hin*dIII window of pKK223-3 after filling in the *Bst*EII cohesive end. The resulting construct, pKR47 (Fig. 2), had eightfold less choline oxidase activity than did pKR32. Deletion of a further 50 bp (pKR46) resulted in complete loss of choline oxidase activity, as demonstrated by subcloning the *Tth*111I-*Hin*dIII fragment of pKR32 into the *Sma*I-*Hin*dIII window of pKK223-3. Thus, these results confine the 5′ end of the *cox* open reading frame to a region bound by the *Bst*EII and *Tth*111I restriction sites (coordinates: kb 2.1 and 2.15, respectively). Deletion of the terminal 500-bp *Pst*I fragment (pKR34) also resulted in loss of choline oxidase activity, thereby indicating that the 3′ end of the *cox* gene lies within

TABLE 1. Effect of carbon source(s) on choline oxidase activity in *A. pascens*[a]

Carbon source 1	Choline oxidase activity (U/mg of total protein ± SE) on carbon source 2			
	None	Glucose	Glycerol	Betaine
None	NA	1.7	3.1 ± 0.9	1.4 ± 0.4
Choline	155.3 ± 17.4	36.4 ± 1.7	90.6 ± 2.1	91.5 ± 15.5
Betaine	1.4 ± 0.4	1.9 ± 0.8	1.4 ± 0.5	NA

[a] *A. pascens* was cultured in Repaske medium containing the macronutrient components (35) and carbon sources (0.2%) as indicated. Results are from triplicate experiments. NA, Not applicable.

kb 3.5 and 4.0. Collectively, these results place the *cox* gene within a 1.9-kb *Bst*EII-*Eco*RI fragment.

Choline oxidase in *A. pascens* is related to carbon metabolism but is not involved in osmotolerance. The metabolic role of choline oxidase was investigated by examining its activity levels when *A. pascens* was cultured in the presence of different carbon sources (Table 1). *A. pascens* was able to utilize choline and betaine as sole carbon sources. Choline induced the enzyme 90-fold (155.3 U/mg) over that observed with a rich carbon source like glucose (1.7 U/mg). A poorer carbon source like glycerol induced choline oxidase by almost twofold (3.1 U/mg) over that observed with glucose. The end product, betaine, did not induce choline oxidase (1.4 U/mg) but repressed the activity by 1.7-fold when added to choline medium (91.5 U/mg). A similar reduction in activity was seen when glycerol was added to choline medium (90.6 U/mg). In contrast, supplementing choline medium with a richer carbon source (glucose) resulted in a larger reduction (fourfold) of choline oxidase activity (36.4 U/mg). These activity levels corresponded with the amount of choline oxidase protein present in the respective cell extracts of *A. pascens* grown in medium containing choline, betaine, glucose, or glucose plus choline (Fig. 3).

The osmoprotective function of choline oxidase was evaluated by culturing *A. pascens* in the presence of an inhibitory amount of NaCl with glucose, glycerol, choline, or betaine as the carbon source. Glucose and glycerol media were supplemented with choline or betaine. Choline and betaine supplements could not enable *A. pascens* to overcome the osmotic stress when glucose or glycerol was provided as the carbon source (Fig. 4).

Expression of the *A. pascens cox* gene in a heterologous host confers osmotolerance. The osmoprotective capacity of choline oxidase in a heterologous system was evaluated by expressing the *A. pascens cox* gene in *E. coli* MC4100 (a deletion mutant defective in oxidation of choline to betaine but proficient in choline uptake [47]). The original *cox*-carrying constructs, pKR1, pKR11, and pKR12, were not maintained by *E. coli* MC4100 when cultured in minimal medium without antibiotic selection. This was found to be a characteristic of pBluescript SK−, the parental vector of these constructs (data not shown). Consequently, the *cox*-containing fragment was subcloned into suitable vectors that were chosen on the basis of stable maintenance by MC4100 during growth in minimal medium. The 4-kb *Hin*dIII-*Bam*HI fragment of pKR12 was subcloned into pHG329 (46), a pBR322 derivative containing the *lac* promoter and multiple cloning sites of pUC19. The resulting construct, pKR26, specified 4.7 U of choline oxidase per mg in MC4100. pKR32 and pKR47, with pKK223-3 (6) as their parental vector (Fig. 2), specified 160.9 and 21.8 U of choline oxidase activity per mg, respectively. Osmotolerance of MC4100 strains possessing these constructs was compared with those of the controls, MC4100(pHG329) (without insert, Cox$^-$), and MC4100 (pLB11) (a construct possessing the *E. coli* choline dehydrogenase and betaine aldehyde dehydrogenase genes [4a]). Both pKR47 and pKR32, which have a 7.5-fold difference in choline oxidase activity, could confer an osmotolerant phenotype to *E. coli* MC4100 when either choline or betaine aldehyde was provided (Fig. 5). Although a similar phenotype was also exhibited by MC4100(pKR26), it was not included in a direct comparison in view of the additional DNA present in the 4-kb insert of pKR26.

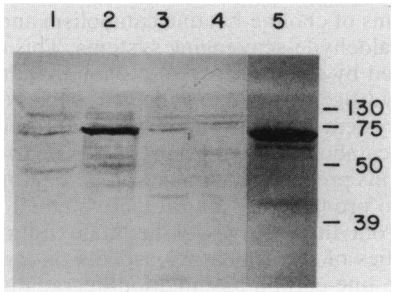

FIG. 3. Induction and repression of choline oxidase synthesis in *A. pascens* by carbon sources. Crude extracts from cells grown in a synthetic medium (Repaske medium) were analyzed in immunoblots with choline oxidase antibodies. Carbon sources (lanes): 1, betaine; 2, choline; 3, choline plus glucose; 4, glucose; 5, purified choline oxidase from *A. globiformis*. The molecular size references are in kilodaltons.

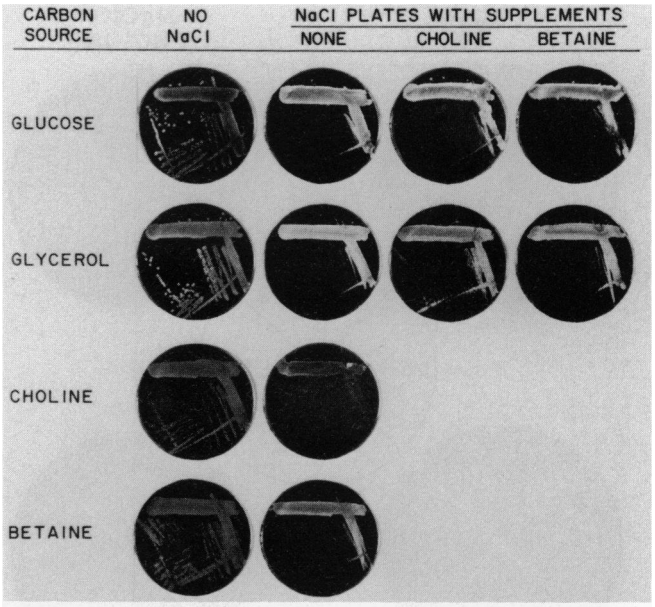

FIG. 4. Choline metabolism does not confer osmotolerance in *A. pascens*. Cell suspensions of *A. pascens* were streaked for single colonies on Repaske medium containing carbon sources (0.2%) as indicated. Choline and betaine when used as supplements were at 1 mM. NaCl was used at 0.6 M. Although 0.8 M NaCl gave the same response as that shown here, higher concentrations were completely inhibitory and lower concentrations promoted higher background growth. Plates were incubated at 30°C for 72 h.

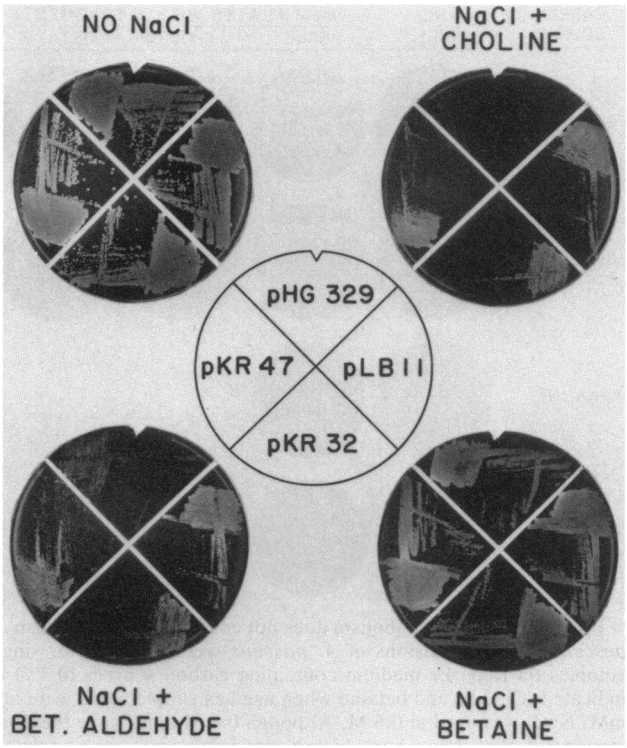

FIG. 5. The *cox* gene of *A. pascens* confers osmotolerance to *E. coli*. *E. coli* MC4100, which is defective in betaine synthesis, was used as the host for all plasmids. The pKR plasmids contain the *cox* gene and pLB11 (4a) carries the betaine biosynthesis genes of *E. coli*. pHG329 (46), a vector without any insert DNA, was used as the negative control; growth of MC4100(pHG329) on NaCl plus betaine aldehyde is due to autooxidation of betaine aldehyde to betaine. M63 medium (28) containing glycerol (0.5%, vol/vol) was used with or without NaCl (0.65 M) and the indicated supplements at 1 mM. The plates were incubated for 48 h at 37°C. The growth response of all four strains on NaCl plates without any supplements was the same as that shown for MC4100(pHG329) on NaCl plus choline.

DISCUSSION

The structural gene for a choline oxidase has been localized to a 1.9-kb *Bst*EII-*Eco*RI fragment. This corresponds well with the requisite size to encode a polypeptide of 66 kDa, the estimated size of choline oxidase from *A. pascens* as determined from immunoblots. Synthesis of a catalytically active choline oxidase in *E. coli* did not detrimentally affect growth. This indicates that *E. coli* can detoxify the additional H_2O_2 produced by choline oxidase, which uses O_2 as an electron acceptor during oxidation of choline to betaine (20). Inclusion of a 0.9-kb segment upstream of the *Bst*EII-*Eco*RI fragment present in pKR47 resulted in a 7.5-fold higher level of choline oxidase activity (pKR32). Since the parental vector for both pKR47 and pKR32 is pKK223-3, which does not provide a translation initiation codon (6), the enhanced expression may be due to posttranscriptional events such as formation of RNA structures that could favor efficient translation when the 0.9-kb leader is present (16).

Expression of choline oxidase in *A. pascens* was positively and negatively regulated by the type and availability of carbon sources. It is likely that *A. pascens*, a soil-borne bacterium (26), has evolved an effective regulatory mechanism for the most economical use of carbon sources in its natural habitat. The relative amounts of choline oxidase protein in cells grown on various carbon sources corresponded with the relative activity levels found when *A. pascens* was cultured in the same media. This is indicative of choline oxidase being regulated at the level of synthesis. The reduction in activity and synthesis of choline oxidase in cells grown in choline plus glucose, in contrast to that in choline-grown cells, suggests catabolite repression. The regulation of choline oxidase in *A. pascens* by carbon source is in agreement with the results of Ikuta et al. (20), who found that levels of choline oxidase activity in *A. globiformis* were higher when an organic medium was supplemented with choline instead of glucose.

Choline and betaine supplements could not enable *A. pascens* to overcome osmotic stress, regardless of the carbon source. Assuming no impairment of uptake in the presence of glucose or glycerol, this suggests that neither choline nor betaine can function as an osmoprotectant in *A. pascens*, unlike in many other bacteria (1, 14, 15, 27, 39, 45). Alternatively, the steady-state concentration of betaine derived by oxidation of choline or from direct uptake of betaine may not be large enough to provide protection from the osmotic stress, due to further utilization of betaine. In *E. coli*, where choline is oxidized to betaine solely to provide an osmolyte to overcome an osmotic stress, betaine synthesis and accumulation are induced by the osmotic stress itself (22, 35) and are independent of the type of carbon source (data not shown). The absence of an osmoprotective function for choline and betaine in *A. pascens* and the modulation of choline oxidase activity by the type of carbon source suggests that in this bacterium the primary role of choline oxidase and its product betaine is in carbon metabolism and not in adaptation to osmotic stress. This contrasts with the observations in another soil bacterium, *Rhizobium meliloti* (45). *R. meliloti* has a choline oxidation pathway that permits catabolism of choline and betaine as carbon and nitrogen sources but promotes betaine accumulation when the bacterium is subjected to osmotic stress. This regulation results from selective repression of enzymes involved in betaine degradation. The dissimilar observations in *R. meliloti* and *A. pascens* may be related to the catabolism of betaine in the two bacteria. In *A. pascens*, methyl groups derived from betaine are oxidized directly to formaldehyde, which may be assimilated by the ribulose monophosphate cycle and serine pathway (24), or the formaldehyde may be oxidized to carbon dioxide by a formaldehyde dehydrogenase (25). *R. meliloti*, on the other hand, utilizes betaine for direct transmethylation of homocysteine to form methionine (45). The potential toxicity of intracellular formaldehyde implies a requirement for appropriate coordination between the regulatory systems of choline-betaine catabolism and the *Arthrobacter* formaldehyde-scavenging systems. This coordination is exemplified by the presence of a choline-induced formaldehyde dehydrogenase in *Arthrobacter* species (25). Such an integrated system might prohibit the preferential inhibition of betaine catabolic enzymes in response to an osmotic stress and thus preclude accumulation of a cytoplasmic pool of betaine to provide an osmoprotective effect. The cloned *cox* gene from this study will facilitate future molecular genetic studies of the regulatory aspects of choline catabolism and the one-carbon assimilation-degradation pathways of *A. pascens*.

Growth of *E. coli* strains expressing the *cox* gene was indistinguishable from that of an *E. coli* strain bearing its native betaine biosynthesis genes. Furthermore, the osmotolerant phenotype required only a relatively low level of choline oxidase activity, as demonstrated by the similar

growth responses of strains possessing pKR47 or pKR32. The osmotolerance response of Cox^+ *E. coli* strains in the presence of choline suggests that choline oxidase can catalyze in vivo both steps involved in betaine biosynthesis. This was further established by fast-atom bombardment mass spectrometry, which detected betaine in cell extracts from various *E. coli* strains expressing *cox* but not from the Cox^- controls (data not shown). Betaine aldehyde could also promote osmotolerance in Cox^+ strains, suggesting in vivo oxidation of an exogenous supply of betaine aldehyde to betaine. The apparent equivalence of the growth responses of Cox^+ strains on choline or betaine aldehyde indicates that the relatively large difference in estimated K_m of choline oxidase for choline (1.2 mM) and betaine aldehyde (8.7 mM) (19) has no effect on the osmotolerant phenotype conferred by *cox* to *E. coli*.

The ability of choline oxidase to catalyze both steps of betaine biosynthesis from choline in vivo questions the reason for an accompanying betaine aldehyde dehydrogenase in *A. pascens* (unpublished data), *A. globiformis* (20), and also in the fungus *Cylindrocarpon didymum* M-1 (29). If choline is utilized as a carbon source, however, the presence of a betaine aldehyde dehydrogenase may be advantageous, given that the K_m for betaine aldehyde of known betaine aldehyde dehydrogenases is 17- to 28-fold lower (22, 29, 31, 34) than the K_m of choline oxidase (8.7 mM [19]). As well, betaine aldehyde dehydrogenase activity can generate NADH or NADPH (22, 29, 31, 34), which may serve in vivo as a source of reductant or be used for ATP synthesis, whereas choline oxidase, which uses O_2 as the primary electron acceptor (20, 33, 49), does not provide this advantage. Therefore, it may be a physiological advantage for a choline-utilizing organism to have a betaine aldehyde dehydrogenase in addition to choline oxidase.

The characteristics of choline oxidase, including it being a soluble and bifunctional enzyme that does not require any cofactors (19, 20), make it a favorable choice to genetically engineer enhanced betaine biosynthesis in heterologous organisms. Although choline oxidase has a covalently attached FAD moiety (19, 33, 49), its assembly into a functional enzyme in the distantly related *E. coli* suggests that it can be modified appropriately in heterologous organisms. Choline dehydrogenase of *E. coli*, the only other choline-oxidizing enzyme for which a gene has been cloned (1, 4a), is a membrane-bound protein that requires an as yet unidentified electron acceptor (22). Thus, the *cox* gene may be suitable for enhancing osmotolerance in biological systems of interest. Native or, if necessary, heterologous H_2O_2-scavenging systems may be used to detoxify the additional H_2O_2 resulting from choline oxidation, and genes encoding catalases and peroxidases have been isolated from a number of sources (13, 38, 41, 50). The cloning and overexpression of the *cox* gene also have applications in clinical biochemistry, where choline oxidase is used for estimation of choline-containing phospholipids in serum (48) and amniotic fluid (10).

ACKNOWLEDGMENTS

G.S. thanks R. Hirji for preliminary screening of some strains for choline oxidase. We are grateful to L. Hogge for fast atom bombardment mass spectrometry analysis, G. S. Stewart for providing pHG329, E. Knapp for illustrations, and the staff at the Animal Care Unit of the University of Saskatchewan for maintaining rabbits.

K.L.R. was supported by scholarships from the Natural Sciences and Engineering Research Council of Canada and the University of Saskatchewan.

REFERENCES

1. **Andresen, P. A., I. Kaasen, O. B. Styrvold, G. Boulnois, and A. R. Strøm.** 1988. Molecular cloning, physical mapping and expression of the *bet* genes governing the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. J. Gen. Microbiol. **134**:1737–1746.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1988. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
3. **Bagnasco, S., R. Balaban, H. M. Fales, Y. M. Yang, and M. Burg.** 1986. Predominant osmotically active organic solutes in rat and rabbit renal medullas. J. Biol. Chem. **261**:5872–5877.
4. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betclach, H. L. Heynecker, H. B. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.

4a. **Boyd, L.** 1990. Ph.D. thesis. University of Saskatchewan, Saskatoon, Canada.

5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Brosius, J., and A. Holy.** 1984. Regulation of ribosomal RNA promoters with a synthetic *lac* operator. Proc. Natl. Acad. Sci. USA **81**:6929–6933.
7. **Brouquisse, R., P. Weigel, D. Rhodes, C. F. Yocum, and A. D. Hanson.** 1989. Evidence for a ferredoxin-dependent choline monooxygenase from spinach chloroplast stroma. Plant Physiol. **90**:322–329.
8. **Burnette, W. N.** 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
9. **Byerrum, R. U., C. S. Sato, and C. D. Ball.** 1956. Utilization of betaine as a methyl group donor in tobacco. Plant Physiol. **31**:374–377.
10. **Campanella, L., M. Tomassetti, G. De Angelis, M. P. Sammartino, and M. Cordatore.** 1987. A new assay for choline-containing phospholipids in amniotic fluid by an enzyme sensor. Clin. Chim. Acta **169**:175–182.
11. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104**:541–555.
12. **Chung, C. T., S. L. Niemela, and R. H. Miller.** 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86**:2172–2175.
13. **Cohen, G., W. Repatz, and H. Ruis.** 1988. Sequence of the *Saccharomyces cerevisiae* CTAI gene and amino acid sequence of catalase derived from it. Eur. J. Biochem. **176**:159–163.
14. **Csonka, L. N.** 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53**:121–147.
15. **Galinski, E. A., and H. G. Trüper.** 1982. Betaine, a compatible solute in the extremely halophilic phototrophic bacterium *Ectothiorhodospira halochloris*. FEMS Microbiol. Lett. **13**:357–360.
16. **Gold, L.** 1988. Posttranscriptional regulatory mechanisms in *Escherichia coli*. Annu. Rev. Biochem. **57**:199–233.
17. **Grumet, R., and A. D. Hanson.** 1986. Genetic evidence for an osmoregulatory function of glycine betaine accumulation in barley. Aust. J. Plant Physiol. **13**:353–364.
18. **Haubrich, D. R., and N. H. Gerber.** 1981. Choline dehydrogenase-assay, properties and inhibitors. Biochem. Pharmacol. **30**:2993–3000.
19. **Ikuta, S., S. Imamura, H. Misaki, and Y. Horiuti.** 1977. Purification and characterization of choline oxidase from *Arthrobacter globiformis*. J. Biochem. **82**:1741–1749.
20. **Ikuta, S., K. Matuura, S. Imamura, H. Misaki, and Y. Horiuti.** 1977. Oxidative pathway of choline to betaine in the soluble fraction prepared from *Arthrobacter globiformis*. J. Biochem. **82**:157–163.
21. **Korstee, G. J. J.** 1970. The aerobic decomposition of choline by

microorganisms. Arch. Mikrobiol. **71**:235–244.
22. **Landfald, B., and A. R. Strøm.** 1986. Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. J. Bacteriol. **165**:849–855.
23. **Le Rudulier, D., A. R. Strøm, A. M. Dandekar, L. T. Smith, and R. C. Valentine.** 1984. Molecular biology of osmoregulation. Science **224**:1064–1068.
24. **Levering, P. R., D. J. Binnema, and W. Harder.** 1981. Enzymatic evidence for a simultaneous operation of two one-carbon assimilation pathways during growth of *Arthrobacter* P1 on choline. FEMS Microbiol. Lett. **12**:19–25.
25. **Levering, P. R., L. Tiesma, J. P. Woldendorp, M. Steensma, and L. Dijkhuizen.** 1987. Isolation and characterization of mutants of the facultative methyltroph *Arthrobacter* P1 blocked in one-carbon metabolism. Arch. Microbiol. **146**:346–352.
26. **Lochhead, A. G., and M. O. Burton.** 1953. An essential bacterial growth factor produced by microbial synthesis. Can. J. Bot. **31**:7–22.
27. **Mackay, M. A., R. S. Norton, and L. J. Borowitzka.** 1984. Organic osmoregulatory solutes in cyanobacteria. J. Gen. Microbiol. **130**:2177–2191.
28. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Mori, N., B. Kawakami, K. Hyakutome, Y. Tani, and H. Yamada.** 1980. Characterization of betaine aldehyde dehydrogenase from *Cylindrocarpon didymum* M-1. Agric. Biol. Chem. **44**:3015–3016.
30. **Morrison, D. A.** 1979. Transformation and preservation of competent bacterial cells by freezing. Methods Enzymol. **68**: 326–331.
31. **Nagasawa, T., Y. Kawabata, Y. Tani, and K. Ogata.** 1976. Purification and characterization of betaine aldehyde dehydrogenase from *Pseudomonas aeruginosa* A-16. Agric. Biol. Chem. **40**:1743–1749.
32. **Nagasawa, T., N. Mori, Y. Tani, and K. Ogata.** 1976. Characterization of choline dehydrogenase from *Pseudomonas aeruginosa* A-16. Agric. Biol. Chem. **40**:2077–2084.
33. **Ohta-Fukuyama, M., Y. Miyake, S. Emi, and T. Yamano.** 1980. Identification and properties of the prosthetic group of choline oxidase from *Alcaligenes* sp. J. Biochem. **88**:197–203.
34. **Pan, S. M.** 1988. Betaine aldehyde dehydrogenase in spinach. Bot. Bull. Acad. Sin. **29**:255–263.
35. **Perroud, B., and D. Le Rudulier.** 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. J. Bacteriol. **161**:393–401.
36. **Pollard, A., and R. G. Wyn Jones.** 1979. Enzyme activities in concentrated solutions of glycinebetaine and other solutes. Planta **144**:291–298.
37. **Repaske, R., and A. C. Repaske.** 1976. Quantitative requirements for exponential growth of *Alcaligenes eutrophus*. Appl. Environ. Microbiol. **32**:585–591.
38. **Roberts, E., and P. E. Kolattukudy.** 1989. Molecular cloning, nucleotide sequence, and abscisic acid induction of a suberization-associated highly anionic peroxidase. Mol. Gen. Genet. **217**:223–232.
39. **Robertson, D. E., D. Noll, M. F. Roberts, J. A. G. F. Menaia, and D. R. Boone.** 1990. Detection of the osmoregulator betaine in methanogens. Appl. Environ. Microbiol. **56**:563–565.
40. **Rothschild, H. A., and E. S. G. Barron.** 1954. The oxidation of betaine aldehyde by betaine aldehyde dehydrogenase. J. Biol. Chem. **209**:511–523.
41. **Sakajo, S., N. Nakamura, and H. Ruis.** 1987. Molecular cloning and nucleotide sequence of full-length cDNA of sweet potato catalase mRNA. Eur. J. Biochem. **176**:159–163.
42. **Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse.** 1988. Lambda ZAP: a bacteriophage lambda expression vector with *in vivo* excision properties. Nucleic Acids Res. **16**:7583–7600.
43. **Skiba, W. E., M. P. Taylor, M. S. Wells, J. H. Mangum, and W. M. J. Awad.** 1982. Human hepatic methionine biosynthesis—purification and characterization of betaine:homocysteine *S*-methyltransferase. J. Biol. Chem. **257**:14944–14948.
44. **Smith, D. E., and P. A. Fisher.** 1984. Identification, developmental regulation, and response to heat shock of two antigenically related forms of a major nuclear envelope protein in *Drosophila* embryos: application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. J. Cell Biol. **99**:20–28.
45. **Smith, L. T., J. A. Pocard, T. Bernard, and D. Le Rudulier.** 1988. Osmotic control of glycine betaine biosynthesis and degradation in *Rhizobium meliloti*. J. Bacteriol. **170**:3142–3149.
46. **Stewart, G. S. A. B., S. Lubinsky-Mink, C. G. Jackson, A. Cassel, and J. Kuhn.** 1986. pHG165: a pBR322 copy number derivative of pUC8 for cloning and expression. Plasmid **15**:172–181.
47. **Styrvold, O. B., P. Falkenberg, B. Landfald, M. W. Eshoo, T. Bjørnsen, and A. R. Strøm.** 1986. Selection, mapping, and characterization of osmoregulatory mutant of *Escherichia coli* blocked in the choline-glycine betaine pathway. J. Bacteriol. **165**:856–863.
48. **Takayama, M., S. Itoh, T. Nagasaki, and I. Tanimizu.** 1977. A new enzymatic method for determination of serum choline-containing phospholipids. Clin. Chim. Acta **79**:93–98.
49. **Tani, Y., N. Mori, K. Ogata, and H. Yamada.** 1979. Production and purification of choline oxidase from *Cylindrocarpon didymum* M-1. Agric. Biol. Chem. **43**:815–820.
50. **Triggs-Raine, B. L., B. W. Doble, M. R. Mulvey, P. A. Sorby, and P. C. Loewen.** 1988. Nucleotide sequence of *katG*, encoding catalase HPI of *Escherichia coli*. J. Bacteriol. **170**:4415–4419.
51. **Weretilnyk, E. A., S. Bednarek, K. F. McCue, D. Rhodes, and A. D. Hanson.** 1989. Comparative biochemical and immunological studies of the glycine betaine synthesis pathway in diverse families of dicotyledons. Planta **178**:342–352.
52. **White, R. F., and A. L. Demain.** 1971. Catabolism of betaine and its relationship to cobalamin overproduction. Biochim. Biophys. Acta **237**:112–119.
53. **Wyn Jones, R. G.** 1984. Phytochemical aspects of osmotic adaptation. Recent Adv. Phytochem. **18**:55–78.
54. **Yancey, P. H., M. E. Clark, S. C. Hand, R. D. Bowlus, and G. N. Somero.** 1982. Living with water stress: evolution of osmolyte systems. Science **217**:1214–1222.