

# L-Serine Catabolism via an Oxygen-Labile L-Serine Dehydratase Is Essential for Colonization of the Avian Gut by *Campylobacter jejuni*

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*Campylobacter jejuni* is a microaerophilic, asaccharolytic bacterium. The identity of the carbon and energy sources used by *C. jejuni* in vivo is unknown, but the genome sequence of strain NCTC11168 indicates the presence of genes for catabolism of a limited range of amino acids, including serine. Specific omission of L-serine from a defined medium containing a mixture of amino acids led to a dramatic decrease in cell yields. As *C. jejuni* does not have a biosynthetic serine requirement, this supports earlier suggestions that L-serine is a preferentially catabolized amino acid. Serine transport was found to be mediated by at least two systems in strain 11168; a high-capacity, low-affinity L-serine-specific system encoded by *Cj1625c* (*sdaC*) and a higher-affinity L-serine/L-threonine system responsible for residual L-serine transport in an *sdaC* mutant. Catabolism of L-serine to pyruvate and ammonia is carried out by SdaA (encoded by *Cj1624c*), which was overexpressed, purified, and shown to be an oxygen-labile iron-sulfur enzyme. L-Serine dehydratase activity in an *sdaA* mutant was reduced 10-fold compared to that in the wild type, but the residual activity (due to the anabolic L-threonine dehydratase) could not support either growth on or utilization of L-serine in defined media. However, although *sdaA* mutants showed no obvious growth defect in complex media, they completely failed to colonize 3-week-old chickens as assayed both by cloacal swabs taken over a 6-week period and by cecal colony counts postmortem. In contrast, the isogenic parent strain colonized chickens to high levels within 1 week of inoculation. The results show that an active SdaA is essential for colonization of the avian gut by *C. jejuni* and imply that catabolism of L-serine is crucially important for the growth of this bacterium in vivo.

*Campylobacter jejuni*, a gram-negative, microaerophilic bacterium, is a leading cause of human enteric disease worldwide and is a major public health and economic burden (7). Although pathogenic in humans, *C. jejuni* is part of the normal commensal flora of many bird species, and ingestion of contaminated poultry is a common route for infection. The pathogenic mechanisms of *C. jejuni* after infection of the human intestinal tract are relatively poorly understood and are generally considered to involve mucosal adherence, host cell invasion, and toxin production (15). The ability to compete for nutrients, particularly carbon and nitrogen sources, is an aspect of pathogen physiology that has received relatively little attention, and it is often assumed that sugars such as glucose are preferred carbon sources in vivo. However, as *C. jejuni* is asaccharolytic (29, 32), alternative carbon sources for growth in the host must be used. Amino acids are likely candidates, as they could potentially be deaminated to a small number of intermediates that can directly feed into the central metabolism, including pyruvate (from serine and alanine), oxaloacetate (from aspartate), and 2-oxoglutarate (from glutamate). Deamination will also generate ammonia, which can be used as a nitrogen source. Nevertheless, there are few data on which

amino acids might be important in vivo. *C. jejuni* is known to grow at the expense of amino acids in vitro and has been shown to utilize serine, aspartate, glutamate, and proline (17). Consistent with these findings, the complete genome sequence of *C. jejuni* NCTC 11168 has revealed the presence of a number of homologues of amino acid-catabolizing enzymes (24).

The study by Leach et al. (17) suggested that, during growth in either complex or defined media, L-serine is preferentially utilized compared to other amino acids. This is not due to a simple biosynthetic requirement for serine, as *C. jejuni* 11168 contains all three genes (*serABC*) necessary for serine biosynthesis from 3-phosphoglycerate (24). An open reading frame (*Cj1624c*) displaying sequence similarity to *sdaA*, which in *Escherichia coli* encodes an L-serine dehydratase, is present in *C. jejuni*. SdaA catalyzes the deamination of serine to pyruvate and ammonia, both of which can be readily assimilated. Although many serine dehydratases also deaminate threonine, the bacterial L-serine dehydratases are much more specific for serine (9). Serine and threonine dehydratases have been purified from many organisms and are usually found to be pyridoxal-5'-phosphate (PLP)-containing enzymes. However oxygen-labile L-serine dehydratases devoid of PLP have been discovered in some strictly anaerobic bacteria, e.g., *Peptostreptococcus asaccharolyticus* (8) and *Clostridium propionicum* (12). These enzymes are inactivated upon exposure to air and can be specifically reactivated by Fe<sup>2+</sup> under anaerobic conditions. The *P. asaccharolyticus* enzyme was found to contain stoichiometric amounts of Fe and inorganic sulfur, sufficient to form one [4Fe-4S] cluster per heterodimer (8). L-Serine dehydrata-

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ses from several other bacteria, including *Clostridium acidurici* (5) and *E. coli* (22), have been recognized to require activation by Fe<sup>2+</sup>, thus making it likely that these enzymes also contain Fe-S centers.

The ability to utilize serine as a carbon or nitrogen source necessitates its efficient transport into the cell. In *C. jejuni* 11168, *sdaA* is the downstream gene of a two-gene operon of which the first gene (*Cj1625c*) encodes a putative serine transporter, homologous to *E. coli* SdaC, an H<sup>+</sup>:L-serine symporter (28). In this study, we have constructed and characterized *sdaA* and *sdaC* mutants of *C. jejuni*, in order to determine the importance of serine as a carbon and nitrogen source in this bacterium. We present evidence that SdaC encodes a high-capacity, low-affinity L-serine-specific uptake system, but an additional high-affinity system still allows residual serine transport in an *sdaC* mutant. The kinetic and biochemical properties of the purified serine dehydratase (SdaA) show that it is an oxygen-labile iron-sulfur protein specific for L-serine deamination. Although the expression of *sdaA* was not necessary for growth in complex media in vitro, SdaA was found to be essential for *C. jejuni* to grow on or utilize L-serine in defined media, as well as for colonization of the ceca of 3-week-old chickens, suggesting a specific requirement for L-serine catabolism for growth in vivo.

#### MATERIALS AND METHODS

**Bacterial strains, media and culture conditions.** *C. jejuni* strains NCTC 11168 and 11168H, a hypermotile variant (13), were routinely cultured at 37°C under microaerobic conditions (10% [vol/vol] O<sub>2</sub>, 5% [vol/vol] CO<sub>2</sub>, and 85% [vol/vol] N<sub>2</sub>) in a MACS growth cabinet (Don Whitley Scientific Ltd., Shipley, United Kingdom) on Columbia agar containing 5% (vol/vol) lysed horse blood and 10 µg each of amphotericin B and vancomycin ml<sup>-1</sup>. Liquid cultures of *C. jejuni* were grown microaerobically with gentle agitation, either in brain heart infusion supplemented with 5% (vol/vol) fetal calf serum (BHI-FCS) or in the defined medium MEM-α (Gibco Ltd.), both containing the above antibiotics and 50 µM FeSO<sub>4</sub>. To select for *C. jejuni* mutants carrying antibiotic-resistant determinants, either kanamycin or chloramphenicol was added to a final concentration of 30 µg ml<sup>-1</sup> each. *E. coli* DH5α was cultured in Luria-Bertani (LB) medium supplemented with appropriate antibiotics at 37°C. For growth assays, *C. jejuni* overnight starter cultures were prepared in BHI before inoculation into fresh BHI or MEM-α to which either 20 mM pyruvate or 20 mM L-serine was added. Growth was monitored at 600 nm in an Amersham Pharmacia Biotech Ultrospec 2000 spectrophotometer. For amino acid utilization studies, MEM-α containing 20 mM pyruvate and either 100 mg of each amino acid liter<sup>-1</sup> or with one specific amino acid omitted was inoculated with *C. jejuni* and was incubated microaerobically for 48 h. Final cell densities were measured at 600 nm.

**DNA isolation and manipulation.** Plasmid DNA was isolated by using the Qiagen Miniprep kit (Qiagen Ltd., Crawley, United Kingdom). *C. jejuni* chromosomal DNA was extracted by using a modified sodium dodecyl sulfate (SDS) lysis procedure (20). Standard techniques were employed for the cloning, transformation, preparation, and restriction analysis of plasmid DNA from *E. coli* (27).

**Inactivation of the *sdaA* and *sdaC* genes.** For inactivation of the *sdaA* gene, a fragment of *Cj1624c* was PCR amplified (forward primer, 5'-ATGCTGTTGGCCTTCTTCTCTCATAAC-3'; and reverse primer, 5'-ATGCAGTGATGGGA GTAATCAATCTTGG-3') from *C. jejuni* 11168 chromosomal DNA and was cloned into pGEM-T Easy (Promega Ltd., United Kingdom). A chloramphenicol acetyltransferase cassette originating from *Campylobacter coli* (33) was then cloned into a unique *NheI* site in *Cj1624c* to generate pJV10. Primers (forward primer, 5'-AGTCTCTAAATGGACTAGTCAGGATAC-3'; and reverse primer, 5'-AGTCTACGAAACCTTGCCAAAATATCAA-3') were designed to amplify a fragment of *Cj1625c* by PCR that was subsequently cloned into pGEM-T Easy. The *aphA-3*-containing *SmaI* fragment of pJMK30 (31), which confers kanamycin resistance, was then cloned into the *BglII* site within the cloned gene to generate pJV11. All antibiotic-resistant constructs were introduced into *C. jejuni* NCTC 11168 or 11168H by electroporation (6). Transformants were selected on Columbia blood agar plates supplemented with the

appropriate antibiotic. Single colonies were then restreaked, and chromosomal DNA was extracted by the microLYSIS kit (Web Scientific Ltd., Crewe, United Kingdom) for screening by PCR, to verify that allelic exchange by double homologous recombination had occurred.

**Preparation of cell extracts and enzyme assays.** Cell extracts of *C. jejuni* were prepared from 300-ml cultures grown for 16 h to the early stationary phase. Cells were harvested by centrifugation (20 min, 4°C, 8,000 × g), washed with 0.1 M Tris-HCl pH 8.0, and then resuspended in 1 ml of the same buffer before sonication as above. The crude extract was clarified by centrifugation (14,000 × g for 30 min), and the supernatant was retained for use on the same day. Serine dehydratase activities were assayed spectrophotometrically in a coupled reaction with L-lactate dehydrogenase by monitoring the decrease in NADH concentration at 340 nm (8). For purified enzyme, assays were performed anaerobically in stoppered glass cuvettes. In these assays, oxygen was removed from all the assay reagents by repeated flushing with nitrogen. Kinetic parameters were determined by an *s/v*-against-*s* plot (11). Cystathionine β-lyase was assayed as described previously (4).

**[<sup>14</sup>C]serine transport assays.** *C. jejuni* cells grown for 16 h in 200-ml BHI-FCS batch cultures were harvested by centrifugation, washed once, and resuspended in 5 ml of 50 mM phosphate buffer, pH 7.4, and kept under microaerobic conditions at 37°C for no longer than 4 h. An aliquot (100 µl) of the cell suspension was added to 4 ml of 50 mM phosphate buffer (pH 7.4) containing 2 mM MgSO<sub>4</sub>, 50 µg of chloramphenicol ml<sup>-1</sup>, and 0.5% (wt/vol) sodium lactate and was allowed to equilibrate by gently stirring at 37°C for 5 min. The assay was initiated by the addition of the specified concentration of [<sup>14</sup>C]-L-serine (1.8 to 2.2 GBq mmol<sup>-1</sup>). Samples (0.4 ml) were withdrawn at the indicated time points, collected by vacuum filtration through membrane filters (0.45-µm pore size), and washed twice with 0.1 M LiCl. Sample filters were then immersed in FluoroSafe 2 scintillation cocktail (BDH Ltd., United Kingdom) and were counted in a Beckman LS 1801 scintillation counter.

For competition experiments, stock solutions of amino acids were diluted into the assay mixture to the specified final concentration, immediately before the addition of [<sup>14</sup>C]-L-serine. In some experiments, the uncoupler, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), was added 10 min before the labeled substrate to a final concentration of 10 µM.

**<sup>1</sup>H NMR spectroscopy.** Culture samples (1.5 ml) were centrifuged to remove cells (13,800 × g, 5 min) and the supernatants were used for nuclear magnetic resonance (NMR) analysis. <sup>1</sup>H NMR was carried out under similar conditions as described previously (18) by using a Bruker AMX500 spectrometer operating at 500 MHz. Spectra were acquired into 8,000 complex points over a spectral width of 12.5 KHz, and the solvent (H<sub>2</sub>O) signal was reduced by presaturation for 2 s. Samples (0.45 ml of culture supernatant plus 0.05 ml of D<sub>2</sub>O) were run in 5-mm-diameter tubes at 30°C. Chemical shifts were established by reference to a standard (trimethylsilylpropionate, TSP; 0 ppm) added to the samples.

**Overexpression and purification of serine dehydratase.** Primers (forward, 5'-GTACCATATGAGTAATTAAGCATTTTT-3'; and reverse, 5'-GTACGG ATCCTTAGCATACTGTTTTAA-3', *NdeI* and *BamHI* restriction sites underlined) were designed to amplify the complete *sdaA* gene from *C. jejuni* 11168 chromosomal DNA by PCR. The resulting amplicon was digested with *BamHI* and *NdeI* and was cloned into similarly restricted pET-14b to give pJV12. This was then transformed into *E. coli* BL21(DE3 pLysS) cells, which were grown aerobically at 30°C in LB medium containing kanamycin (30 µg ml<sup>-1</sup>) and chloramphenicol (30 µg ml<sup>-1</sup>) to an optical density at 600 nm of 0.6, before induction by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Induced cells were then grown for a further 5 h before harvesting by centrifugation (20 min, 4°C, 8,000 × g). Cell pellets were resuspended in 20 mM potassium phosphate buffer, pH 7.4, containing 0.5 M NaCl, and were sonicated (6 × 10-s bursts, 12-µm amplitude, Soniprep 150; MSE), and the sonicate was centrifuged at 14,000 × g for 30 min at 4°C. The His<sub>6</sub> affinity tag permitted the purification of the enzyme from the supernatant by affinity chromatography on nickel-nitrilotriacetic acid resin according to the protocol of the HisTrap kit (Amersham Pharmacia Biotech Ltd., Bucks, United Kingdom). The protein was eluted from the resin by a step gradient from 100 to 300 mM imidazole in 20 mM potassium phosphate buffer, pH 7.0, containing 0.5 M NaCl, before desalting on an Econo-Pac 10DG column (Bio-Rad Ltd., United Kingdom) according to the manufacturer's instructions. Molecular mass determinations of water-dialyzed SdaA by electrospray mass spectrometry were performed by S. Thorpe, Department of Chemistry, University of Sheffield, with a VG Bio Q spectrometer. Native molecular mass was determined by gel filtration chromatography in 10 mM Tris-HCl, pH 8.0, containing 200 mM NaCl on a calibrated Superdex-200 column attached to a Pharmacia fast protein liquid chromatography system.

**Chicken colonization studies.** Day-of-hatching specific-pathogen-free (SPF) Rhode Island Red chicks were obtained from the Poultry Production Unit,

Institute for Animal Health, Compton, United Kingdom. All the birds were inoculated orally with 0.1 ml of *Campylobacter*-free adult gut flora preparations. This was produced by static culture of 1 g of cecal contents in 10 ml of LB broth for 23 h at 37°C, taken from a 50-week-old SPF chicken immediately after the bird had been killed. The birds thus inoculated were housed under appropriate SPF conditions until 3 weeks of age when they were used in colonization trials. Birds were fed ad libitum on a vegetable-based diet (Special Diet Services, Manea, Cambridgeshire, United Kingdom). Groups of 20 3-week-old birds with the developed gut flora were inoculated orally with 0.1 ml of culture containing  $10^7$  CFU of the desired *Campylobacter* strain. Sterile cloacal swabs were taken from birds unidirectionally, and fecal secretion was assessed by both direct counts and enrichment counts as follows: cloacal swabs were taken at weekly intervals and were mixed into 1 ml of modified Exeter broth (2). Swabs were streaked in a standard manner onto *Campylobacter* blood-free selective agar (CCDA and CM739 [Oxoid Ltd., United Kingdom] containing CCDA selective supplement SR155; Oxoid Ltd.), and colony counts were scored positive ( $>1$  CFU, direct counts) or negative after 2 days of incubation. Swabs were returned to the enrichment medium and were incubated under microaerobic conditions at 37°C. After 2 days the enrichment cultures were checked for *Campylobacter* by plating on *Campylobacter* blood-free agar. These were scored as positive or negative results and are referred to as enrichment counts. At 6 weeks postinfection, the birds were killed humanely and postmortem bacterial counts were determined from the cecal contents of five birds from each group. Samples were diluted in phosphate-buffered saline) and were immediately plated on *Campylobacter* blood-free agar. All postmortem counts are expressed as number of CFU per gram.

## RESULTS

**Effect of omission of L-serine from growth media.** Specific amino acids that enhance the growth of *C. jejuni* 11168 in vitro were identified by measuring final cell densities after 48 h growth in MEM- $\alpha$  containing 20 mM pyruvate and either a mixture of all 20 amino acids (control) or with individual amino acids omitted. In a typical experiment, single omission of Ala, Asp, Glu, Gly, His, Ile, Lys, Phe, Pro, Thr, Trp, or Tyr resulted in a  $<25\%$  decrease in final yields, whereas omission of methionine or cysteine resulted in moderate growth inhibition (to 39 or 32% of the final optical density of the control, respectively). However, the specific absence of L-serine in the medium was most detrimental to growth, resulting in a 54% lower final cell yield compared to the result for the complete amino acid control.

**Construction of *sdaA* and *sdaC* mutants: *sdaC* encodes a low-affinity L-serine specific transporter.** *C. jejuni sdaA* and *sdaC* mutants were generated by the insertion of nonpolar antibiotic resistance cassettes (containing an internal promoter but lacking transcriptional terminators) into unique restriction sites within the cloned genes. The resulting constructs were electroporated into *C. jejuni* 11168, generating chromosomal null mutants of *sdaA* and *sdaC*. The correct construction of the mutants was verified by PCR amplification of the target genes, yielding fragment sizes consistent with the insertion of the 0.75- and 1.5-kb chloramphenicol and kanamycin resistance cassettes, respectively (data not shown).

The wild type and *sdaA* and *sdaC* mutants were assayed for [ $^{14}$ C]L-serine uptake. At an external concentration of 10  $\mu$ M, the *sdaC* mutant exhibited a significantly lower rate of [ $^{14}$ C]L-serine uptake ( $143 \pm 2$  pmol  $\text{min}^{-1}$  mg of protein $^{-1}$ ) than did the wild type ( $1,073 \pm 168$  pmol  $\text{min}^{-1}$  mg of protein $^{-1}$ ). In contrast, the *sdaA* mutant did not display any deficiency in [ $^{14}$ C]L-serine transport ( $1,287 \pm 250$  pmol  $\text{min}^{-1}$  mg of protein $^{-1}$ ). The concentration dependence of [ $^{14}$ C]L-serine transport was examined in the wild type and the *sdaC* mutants. In

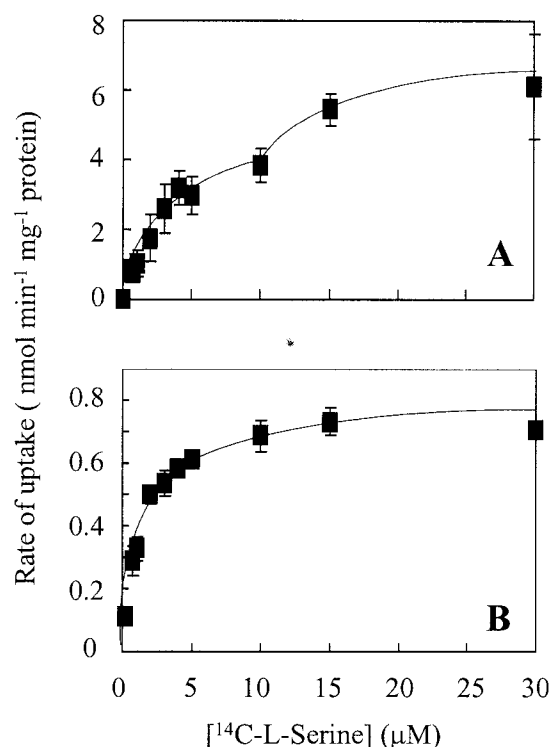


FIG. 1. Concentration dependence of [ $^{14}$ C]L-serine uptake by *C. jejuni* 11168 wild-type (A) and *sdaC* (B) strains. Each data point represents the mean of assays on two different batches of cells, and the error bars represent the standard deviation.

the wild type (Fig. 1A), over a [ $^{14}$ C]L-serine concentration range of 0.25 to 30  $\mu$ M, the rate of [ $^{14}$ C]L-serine uptake was biphasic, a pattern indicative of the presence of at least two transport systems with different affinities for serine. The higher-affinity system had a  $K_m$  value of about 2.5  $\mu$ M, but an accurate estimate of the affinity of the lower-affinity system could not be obtained. In the *sdaC* mutant (Fig. 1B), the [ $^{14}$ C]serine uptake rates, at all concentrations tested, were significantly lower than those measured in the wild type and the *sdaA* mutant. The data could be fitted to a monophasic hyperbolic saturation curve with a single  $K_m$  of 1.4  $\mu$ M. Thus, the data indicate that *sdaC* encodes the low-affinity serine transporter and that the remaining transport activity in the *sdaC* mutant is due to a separate high-affinity system.

**Substrate specificity of serine transport.** In wild-type cells transport of low (5  $\mu$ M) or high (30  $\mu$ M) concentrations of L-serine was strongly inhibited by the protonophore FCCP, which provides a route for the dissipation of the proton electrochemical gradient. The specificity of [ $^{14}$ C]L-serine transport was investigated by examining the ability of other amino acids, including L-isoleucine, L-leucine, L-valine, L-glycine, L-threonine, L-proline, L-glutamine, L-histidine, and D-serine, to inhibit [ $^{14}$ C]L-serine accumulation. To examine high-affinity transport, the amino acids were added at a concentration of 50  $\mu$ M, and [ $^{14}$ C]L-serine was present at a concentration of 5  $\mu$ M. In wild-type cells, [ $^{14}$ C]L-serine transport was not significantly inhibited by any of the amino acids tested, with the exception (as expected) of unlabeled L-serine, which resulted in 84%

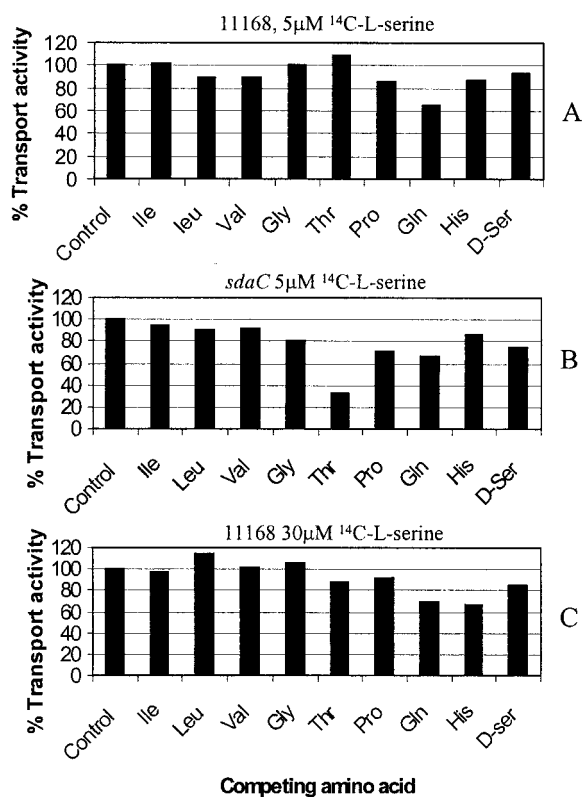


FIG. 2. Effect of competing amino acids on [ $^{14}\text{C}$ ]L-serine uptake in the wild type (A and C) and *sdaC* mutant (B). High-affinity uptake was examined by using 5  $\mu\text{M}$  [ $^{14}\text{C}$ ]L-serine, with the addition of 50  $\mu\text{M}$  competing unlabeled amino acid as indicated, while low-affinity uptake was examined with 30  $\mu\text{M}$  [ $^{14}\text{C}$ ]L-serine and 300  $\mu\text{M}$  competing amino acid as indicated. Inhibition is shown as the percentage of the uptake rate observed in the control incubation, where no competing amino acid was added. The data shown are from a single representative experiment; other experiments gave similar results.

inhibition (Fig. 2A). However, in the *sdaC* mutant, strong competition was observed with 50  $\mu\text{M}$  L-threonine (Fig. 2B) with a 68% inhibition of [ $^{14}\text{C}$ ]serine transport. The substrate specificity of low-affinity serine transport in the wild type was also investigated, by performing competition experiments at a [ $^{14}\text{C}$ ]L-serine concentration of 30  $\mu\text{M}$ . When present at a concentration of 300  $\mu\text{M}$ , none of the amino acids tested significantly inhibited [ $^{14}\text{C}$ ]serine transport (Fig. 2C). Thus, low-affinity serine transport (encoded by *sdaC*) is specific for L-serine, while the additional high-affinity transporter revealed in the *sdaC* mutant may also transport L-threonine. The presence of both transporters probably masks threonine inhibition of transport of the high-affinity system in wild-type cells. As neither L-isoleucine, L-leucine, or L-valine inhibited the low-affinity transport of [ $^{14}\text{C}$ ]L-serine into the wild type (Fig. 2C), it is unlikely that the branched-chain amino acid (LIV) transport system contributes to uptake of serine at the concentrations used.

**Requirement of serine dehydratase activity for growth and utilization of L-serine.** The ability of MEM- $\alpha$ , supplemented with 20 mM L-serine or 20 mM pyruvate, to support the growth of the wild type and the *sdaA* mutant is illustrated in Fig. 3. The wild type grew well with either L-serine or

pyruvate as the primary carbon source in MEM- $\alpha$ . However, although the final cell yield of the *sdaA* mutant on pyruvate was comparable to that of the wild type, the *sdaA* mutant did not grow on L-serine (Fig. 3A). This indicates that serine dehydratase is essential for growth with serine but not pyruvate as the major carbon source.  $^1\text{H}$  NMR spectroscopy of culture supernatants was employed to directly measure the utilization of serine in wild-type and *sdaA* mutant cells grown with 20 mM L-serine in MEM. The NMR spectra presented in Fig. 3B and C show that, in contrast to the wild type, the *sdaA* mutant was unable to metabolize serine to any significant extent. In contrast to the obvious phenotype in MEM, no differences in either growth rate or final cell yields were observed between the wild type and the *sdaA* mutant when grown in the complex BHI-FCS medium (data not shown).

***sdaA* encodes an L-serine specific dehydratase that is PLP independent.** Serine dehydratase enzyme activity was decreased 10-fold in cell extracts of the *sdaA* mutant compared to the wild-type parent strain (Table 1). The residual activity observed in the *sdaA* mutant could be due to the *metC* product, cystathionine  $\beta$ -lyase, which has been reported to contribute to serine deamination in vivo (4). However, *C. jejuni* contains only two pseudogene fragments encoding a putative cystathionine  $\beta$ -lyase (*Cj1392/1393*), and no activity could be detected when wild-type or *sdaA* mutant cell extracts were assayed for this enzyme. Another potential serine-deaminating enzyme is threonine dehydratase, of which there are two types, catabolic and biosynthetic. These two enzymes are readily distinguished by the fact that the biosynthetic enzyme is sensitive to inhibition by isoleucine. A gene encoding a biosynthetic threonine dehydratase, *ilvA* (*Cj0828c*), was identified from the genome sequence of strain 11168 (24). Threonine dehydratases are PLP-dependent enzymes, which can be inactivated by carbonyl reagents, including phenylhydrazine and hydroxylamine, that specifically interact with the Schiff base to which the prosthetic group is bound (9). The residual serine dehydratase activity detected in the *sdaA* mutant was completely inhibited by 10 mM phenylhydrazine or 1 mM hydroxylamine (Table 1), thus confirming that a PLP-dependent enzyme is responsible for this activity. Moreover, the activity was completely abolished in the presence of 1 mM isoleucine. Thus, the low residual activity in the *sdaA* mutant can be ascribed to the biosynthetic threonine dehydratase. Importantly, wild-type L-serine dehydratase activity was not significantly changed in the presence of either phenylhydrazine or hydroxylamine (Table 1), suggesting that the *C. jejuni* SdaA enzyme is devoid of PLP. The wild-type activity was weakly inhibited by D-serine and L-threonine but was more strongly inhibited by L-cysteine (Table 1). We attempted to complement the 11168 *sdaA* mutant by using a “knock-in” approach, i.e., by integrating a plasmid containing a wild-type copy of the *sdaA* gene at the *rdxA* locus that, when disrupted, gives rise to metronidazole resistance, thus allowing positive selection for integrants. However, while such integrants were obtained, as proved by PCR analysis, none was complemented for SdaA enzyme activity.

Unexpectedly, serine dehydratase activity due to SdaA (as assayed in the wild type in the presence of 1 mM hydroxylamine) was found to be completely undetectable in the *sdaC* mutant (data not shown). This indicates a polar effect of the *sdaC* mutation on *sdaA*, although the antibiotic resistance car-

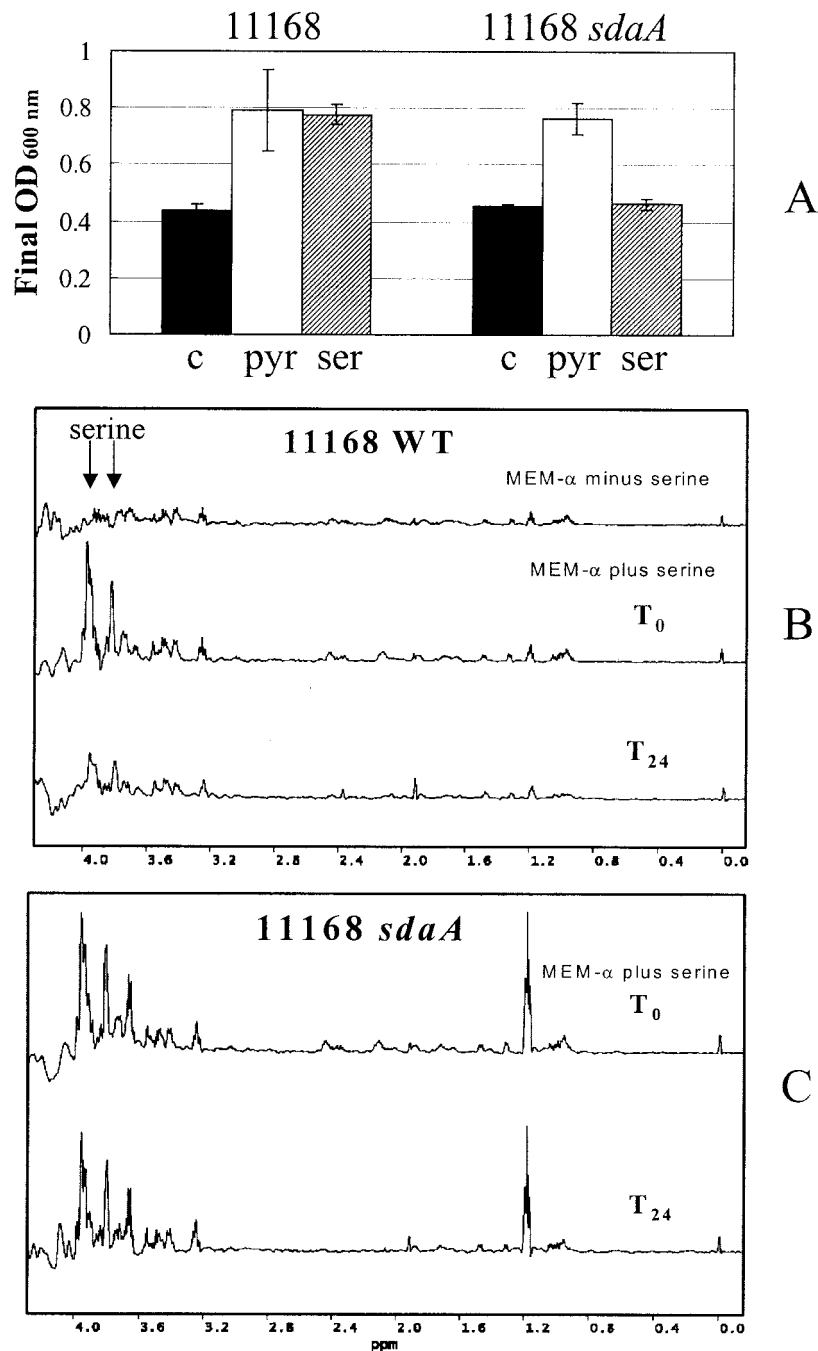


FIG. 3. (A) Growth of the *C. jejuni* wild type and *sdaA* mutant in MEM- $\alpha$ , in the presence and absence of an added carbon source. Solid bars (c), no added carbon source; open bars (pyr), 20 mM sodium pyruvate; and hatched bars (ser), 20 mM L-serine. Strains were pregrown overnight in BHI-FCS, harvested, washed, and resuspended in MEM- $\alpha$  before being inoculated into prewarmed MEM- $\alpha$ . It should be noted that, in MEM- $\alpha$  alone (solid bars), a basal level of growth occurs owing to the presence of amino acids in the medium (starting optical density at 600 nm [OD<sub>600 nm</sub>] was 0.28). The data represent the mean of two separate experiments  $\pm$  standard deviation. (B and C) Analysis of L-serine utilization by *C. jejuni* wild-type (B) and *sdaA* mutant (C) strains conducted by using proton NMR spectroscopy. Cells were grown with 20 mM L-serine in MEM- $\alpha$ . Culture supernatants for each strain were collected before (T<sub>0</sub>) and after 24 h (T<sub>24</sub>) of inoculation. The serine proton resonances at a chemical shift of 3.7 to 3.9 ppm are indicated with arrows, as determined from the spectrum for MEM- $\alpha$  alone. The chemical shift reference used was 1 mM trimethylsilylpropionate (0 ppm), which was included in all the samples. The identity of the resonance at 1.2 ppm in the *sdaA* samples was not determined.

tridge within the *sdaC* gene is devoid of known transcriptional terminators and was present in the same transcriptional orientation as the *sdaC* gene.

**Purification and catalytic properties of L-serine dehydratase.** In order to determine the biochemical properties of

the *C. jejuni* serine dehydratase, Cj1624 was overexpressed in *E. coli* BL21(DE3) as a histidine-tagged protein, followed by purification using Ni-affinity chromatography. Although serine dehydratase in *C. jejuni* cell extracts was stable in the presence of air, the purified enzyme was inactivated upon exposure to

TABLE 1. Effect of phenylhydrazine, hydroxylamine, and amino acids on serine dehydratase activity in cell extracts of the *C. jejuni* 11168 wild type and *sdaA* mutant<sup>a</sup>

| Addition              | L-Serine dehydratase activity (nmol min <sup>-1</sup> mg of protein <sup>-1</sup> ) for: |                          |
|-----------------------|--|--------------------------|
|                       | 11168 wild-type  | 11168 <i>sdaA</i> mutant |
| None                  | 434 ± 42   | 40 ± 9                   |
| 10 mM phenylhydrazine | 402 ± 81   | 0                        |
| 1 mM hydroxylamine    | 430 ± 16   | 0                        |
| 1 mM isoleucine       | 431 ± 62   | 0                        |
| 100 mM D-serine       | 260 ± 93   | ND                       |
| 100 mM L-threonine    | 194 ± 11   | ND                       |
| 50 mM L-cysteine      | 22 ± 9   | ND                       |

<sup>a</sup> The mean serine dehydratase activity ± standard deviation is indicated for experiments performed in duplicate. ND, not determined.

air (see below), which necessitated purification to be carried out under strictly anaerobic conditions. The enzyme was purified ninefold to apparent homogeneity in a single step, to a specific activity of 115 U mg of protein<sup>-1</sup>, with the yield being almost 100%. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme gave a single band of approximately 49 kDa (Fig. 4A). The predicted molecular mass of the overexpressed SdaA plus the His-tag residues is 52,032 Da; electrospray mass spectroscopy gave a molecular mass of 51,758 Da. The apparent molecular mass of the native enzyme was determined as 123 ± 3 kDa by gel filtration on a calibrated Superdex-200 column (Fig. 4B), which suggests that the native enzyme is probably a homodimer.

The catalytic properties of SdaA were determined under anaerobic conditions immediately following the purification procedure. The enzyme was highly specific for L-serine, with a  $K_m$  of 14.6 ± 3.4 mM and a  $V_{max}$  of 403 ± 35 μmol min<sup>-1</sup> mg of protein<sup>-1</sup>. Neither L-threonine, D-serine, nor L-cysteine was detectably deaminated (data not shown). D-Serine was slightly inhibitory to L-serine dehydratase activity (in one set of experiments, 52.1 ± 6 U of L-serine dehydratase activity mg of protein<sup>-1</sup> was observed in the presence of 100 mM D-serine, compared to 73.5 ± 4.3 U mg of protein<sup>-1</sup> in its absence), while no L-serine dehydratase activity at all was detected in the presence of 50 mM L-cysteine. This is consistent with the properties of the activity observed in cell extracts (Table 1).

**SdaA is an iron-sulfur enzyme: reversible inactivation of the purified enzyme in air.** The pure enzyme was yellow-brown. Its UV spectrum revealed, in addition to the protein peak at 280 nm, two broad shoulders at 300 to 350 nm and at 420 nm (Fig. 5A and inset). These spectral features are characteristic of enzymes containing iron-sulfur clusters. Purified L-serine dehydratase was inactivated, with a half-life of approximately 1.5 h, upon exposure to air (Fig. 5B). This was associated with the loss of the 300- to 350-nm and 420-nm shoulders in the UV spectrum (Fig. 5A). The inactivated enzyme could be reactivated somewhat with Fe<sup>2+</sup> and dithiothreitol (DTT) under strict anaerobic conditions (Fig. 5C), with activity being restored to about 60% of the activity of enzyme stored under anaerobic conditions. The reactivation was highly specific for Fe<sup>2+</sup>, as other metal ions such as Fe<sup>3+</sup>, Cu<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, and Ni<sup>2+</sup> had no effect (data not shown). Similarly, serine

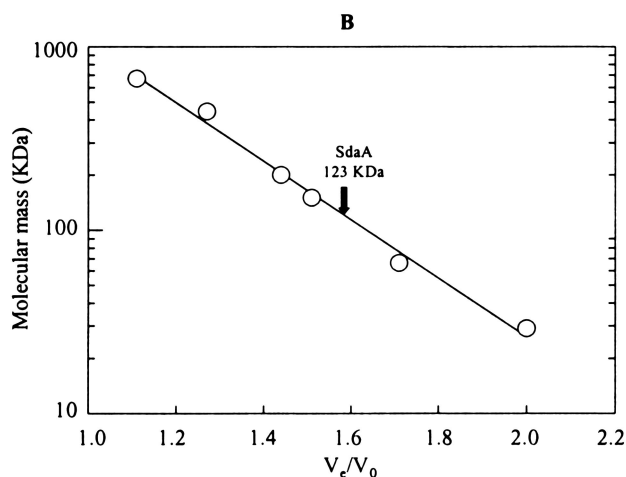
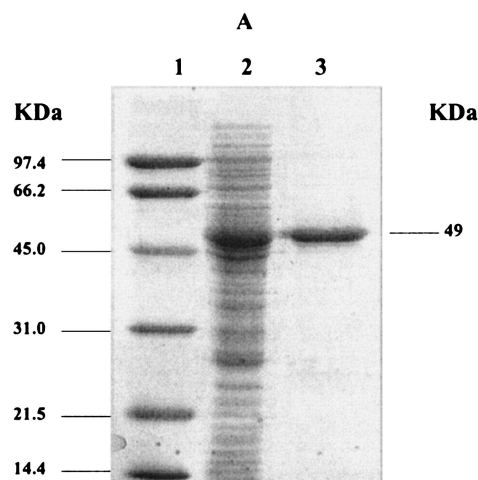


FIG. 4. (A) Purification of recombinant *C. jejuni* serine dehydratase. Lane 1, molecular mass markers (phosphorylase B, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa). Lane 2, 15 μg of cell extract protein from BL21(DE3 pLysS pJV12) after IPTG induction for 5 h. Lane 3, purified L-serine dehydratase (3 μg) following chromatography on nickel-nitrilotriacetic acid resin. (B) Determination of the native molecular weight of purified SdaA by gel filtration on Superdex-200. The column was calibrated by using thyroglobulin (669 kDa), horse spleen apoferritin (443 kDa), sweet potato amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and bovine erythrocyte carbonic anhydrase (29 kDa).  $V_e$ , protein elution volume;  $V_0$ , column void volume (as determined with blue dextran).

dehydratase activity could not be restored by DTT alone, in the absence of Fe<sup>2+</sup> (data not shown).

***C. jejuni sdaA* mutants do not colonize the avian intestinal tract.** An *sdaA* mutant was employed to test the importance of serine dehydratase in the in vivo colonization of *C. jejuni* in a chicken model. Due to the lack of SdaA activity in the *sdaC* mutant, we could not test the in vivo role of SdaC separate from SdaA. The *sdaA* mutation was introduced into *C. jejuni* strain 11168H, a hypermotile variant previously described by Karlyshev et al. (13). This strain has been found to be colonization proficient to high levels in the chick intestinal tract

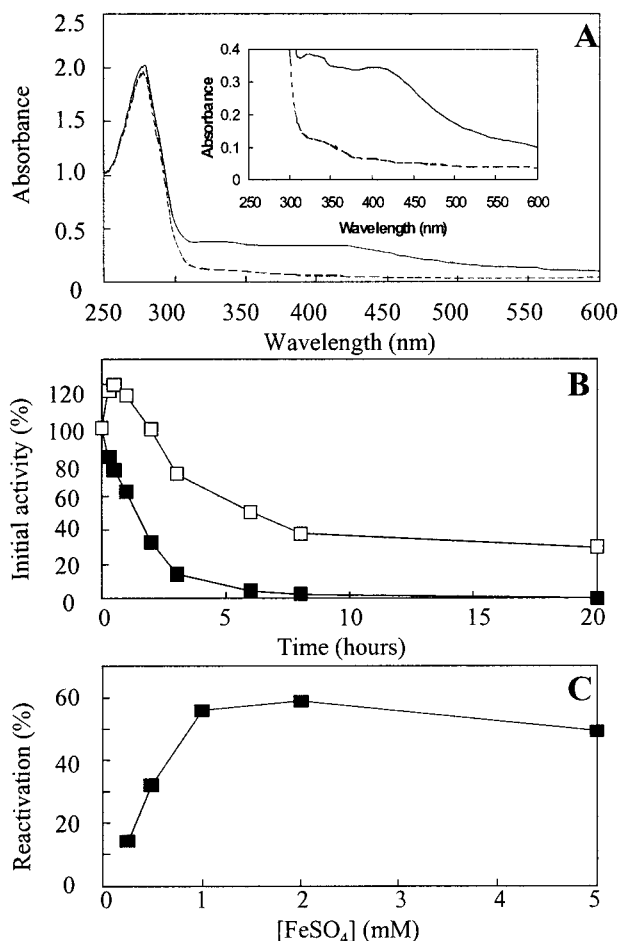


FIG. 5. SdaA is an oxygen-labile iron-sulfur enzyme. (A) UV/visible spectra of active pure L-serine dehydratase (solid line) measured under anaerobic conditions and air-inactivated L-serine dehydratase (broken line), showing loss of absorbance at 420 nm (characteristic of iron-sulfur enzymes) upon exposure to air. (B) Inactivation kinetics of L-serine dehydratase in air. SdaA in 20 mM potassium phosphate buffer, pH 7.0, was incubated at 4°C under anaerobic conditions (□) or in the presence of air (■). Samples were withdrawn at the indicated time points and were assayed under anaerobic conditions for L-serine dehydratase activity as described in Materials and Methods. The data are from a single assay but are representative of two independent experiments. (C) Reactivation of L-serine dehydratase with Fe<sup>2+</sup> and DTT. Active L-serine dehydratase in 50 mM potassium phosphate buffer, pH 7.0, was exposed to air for 24 h at 4°C until no activity could be detected. The enzyme was then transferred to an anaerobic chamber and was incubated for 1 h at 4°C with FeSO<sub>4</sub> at the specified concentrations and with 10 mM DTT in each case. The resulting L-serine dehydratase activities were measured under anaerobic conditions. Reactivation is expressed as a percentage of the activity of the enzyme stored under anaerobic conditions for the duration of the inactivation in air and subsequent reactivation with Fe<sup>2+</sup> and DTT.

(M. A. Jones, K. Marston, B. Wren, and P. A. Barrow, unpublished data). *C. jejuni* 11168, in contrast, has a persistent low-level infection and undergoes adaptation to be a high-level colonizer of chickens only over a period of weeks (Jones, Marston, and Barrow, unpublished). PCR was used to verify the correct construction of the 11168H *sdaA* mutant (data not shown), and we confirmed that this mutant had the expected growth defect on L-serine and was devoid of SdaA-mediated

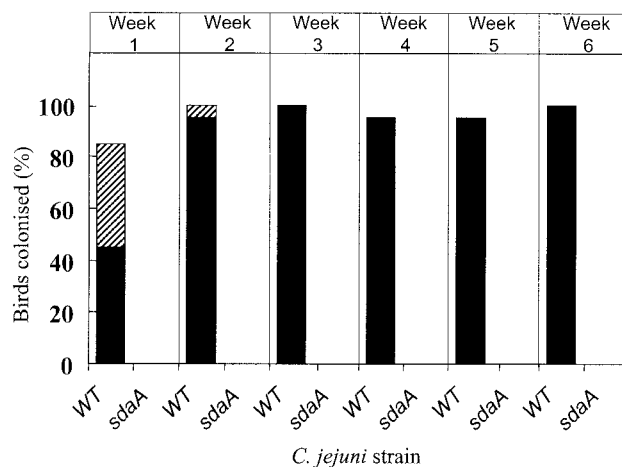


FIG. 6. Colonization of 3-week-old chickens by *C. jejuni* 11168H (WT) and 11168H *sdaA*. Groups of 20 chickens with *Campylobacter*-free adult gut flora were inoculated with 10<sup>7</sup> CFU of either strain. At weekly intervals, cloacal swabs were taken and the extent of colonization was determined. The data are expressed as a dual percentage of the number of chickens colonized, as assessed by both direct counts (solid black bars) and enrichment counts (hatched bars). See Materials and Methods for further details.

serine dehydratase enzyme activity. At weekly intervals after inoculation, the colonization data from cloacal swabs were expressed as a dual percentage of the groups of birds colonized as assessed by both direct counts and growth after incubation in an enrichment medium (Fig. 6). This semiquantitative method has been previously described in genetic mapping studies for colonization of chickens by *Salmonella enterica* serovar Typhimurium (1, 30) and has recently been used in studies on avian resistance to *Campylobacter* colonization (Y. Boyd, Jones, and Barrow, unpublished). Figure 6 clearly shows that the 11168H *sdaA* mutant failed to colonize the avian host to a detectable level when compared to the parent strain, which within 1 week had colonized 90 to 100% of the birds inoculated. In addition to the cloacal swab data, cecal content counts of sacrificed birds at 6 weeks postinfection showed that strain 11168H colonized to a level of  $2.81 \times 10^8 \pm 1.92 \times 10^8$  CFU g<sup>-1</sup>, but the counts for the birds inoculated with the 11168H *sdaA* mutant were below detectable limits (<100 CFU g<sup>-1</sup>).

## DISCUSSION

In this study, the specific omission of L-serine from a defined growth medium resulted in the largest decrease in cell yields of all the amino acids tested, despite the presence of all three genes (*serA*, *serB*, and *serC*) required for serine biosynthesis (24). In the absence of serine, both the growth rate and final cell density were lowered. Previous studies had suggested that serine is preferentially utilized when *C. jejuni* is grown with a mixture of amino acids (17). Thus, serine catabolism appears to be especially important in the normal physiology of *C. jejuni*, presumably because the two products of serine deamination (pyruvate and ammonia) provide forms of carbon and nitrogen that can feed directly into the central metabolism. Pyruvate has been shown to be one of the best carbon sources for *C. jejuni*, as well as being the starting point for gluconeogenesis (32), and

ammonia can be incorporated via the glutamine synthetase/glutamate:2-oxoglutarate aminotransferase route (24).

Serine transport in *C. jejuni* is carried out by at least two systems, a low-affinity L-serine-specific transporter encoded by *sdaC* and a high-affinity transporter that was revealed by analysis of the residual transport kinetics of the *sdaC* mutant. The hydropathy profile (16) of SdaC shows that it contains 10 or 11 potential membrane-spanning segments and that its overall topology is similar to the *E. coli* SdaC protein and other members of the hydroxy/aromatic amino acid permease family (26). The identity of the high-affinity transporter is unknown, but the competition studies suggest that it could be a dual serine-threonine transporter. This type of system is known in other bacteria, for example, the Na<sup>+</sup>-coupled threonine-serine uptake system in *E. coli* (10).

Several lines of evidence show that the *sdaA* gene encodes an L-serine dehydratase that is devoid of PLP. The enzyme lacks the consensus sequence centered around a lysyl residue to which PLP binds via a Schiff base (23), in addition to lacking the glycine-rich region reported for all PLP-dependent enzymes (19). Moreover, the carbonyl reagents phenylhydrazine and hydroxylamine did not significantly affect enzyme activity. The absorption spectrum of the purified protein was, however, typical of that for an iron-sulfur protein, with broad peaks at 300 to 350 and 420 nm. Consistent with this, the *C. jejuni* SdaA sequence contains four conserved cysteine residues, which could coordinate a [4Fe-4S] cluster. Moreover, both the spectral features and enzyme activity were lost upon exposure to air, but activity could be restored by treatment with ferrous iron and a reducing agent. These properties are similar to those exhibited by the L-serine dehydratases of many anaerobes, e.g., *P. asaccharolyticus*, which are PLP-independent, iron-sulfur-containing enzymes (8, 9). This family of enzymes share mechanistic similarities with the dehydration of citrate by aconitase (9), but they are generally highly specific for L-serine deamination. Although the purified *C. jejuni* enzyme did not detectably deaminate L-threonine, this amino acid and D-serine were weak inhibitors of both the purified enzyme and the activity in cell extracts. L-Cysteine inhibition was much more potent, and studies of the SdaA enzymes from several anaerobes have shown both D-serine and particularly L-cysteine to be competitive inhibitors (8, 12). The employment of an oxygen-labile dehydratase for serine catabolism is another example of the use by *C. jejuni* of a type of enzyme more commonly found in anaerobic bacteria than aerobes. Other examples include the pyruvate and 2-oxoglutarate:acceptor oxidoreductases, which are used instead of the 2-oxoacid dehydrogenase multienzyme complexes as found in most aerobes (14).

Mutation of *sdaA* resulted in a ~10-fold reduction in the activity of serine dehydratase in cell extracts, and the residual activity was fully sensitive to inhibition by the carbonyl reagents phenylhydrazine and hydroxylamine. This, coupled with the inhibition of the residual activity by isoleucine, strongly indicates that the PLP-dependent biosynthetic L-threonine dehydratase (IlvA; Cj0828c) is responsible for the low but detectable rate of serine deamination in the mutant. However, this residual activity was unable to support growth of the mutant on L-serine in a minimal medium and there was no evidence from <sup>1</sup>H NMR spectroscopy for significant utilization of L-serine by intact cells. Despite this clear phenotype and the apparent

preference of *C. jejuni* for serine catabolism demonstrated by the study of Leach et al. (17) and in this study by the results of amino acid omission, growth of the *sdaA* mutant in a complex medium (BHI-FCS) was indistinguishable from that of the parent strain. This indicates that an inability to deaminate serine does not result in a growth disadvantage when a larger array of carbon sources in addition to amino acids are available. It was therefore considered important to determine if the ability of this mutant to colonize an animal model was compromised, in order to obtain information on whether serine is available and is preferentially utilized in vivo.

A variety of avian hosts are important natural reservoirs of *C. jejuni*, but the chicken is the most relevant in terms of the human food chain. Various chick infection models have been used very successfully in studies comparing colonization of different strains of wild-type or mutant bacteria (3, 21, 34), but in the field, birds are naturally colonized at 2 or 3 weeks of age. In this study, we have used 3-week-old chickens for colonization trials with a hypermotile variant of strain 11168 (13), which resulted in high-level colonization within 1 week, and this level of colonization was maintained for the duration of the experiments. The complete lack of colonization of the isogenic *sdaA* mutant, evidenced by the repeated lack of recovery of viable bacteria by either enrichment from cloacal swabs or by cecal content counts at the end of the experiment, clearly shows that serine dehydratase encoded by *sdaA* is an essential colonization factor for *C. jejuni*. The *sdaA* mutant shows only one phenotype relating to L-serine utilization; it has a growth rate identical to that of the parent strain in complex media and we have detected no increased sensitivity to oxidative stress, for example, that might lead to decreased survival in the host. Thus, the data suggest that a source of serine is readily available to the bacteria and that its catabolism via SdaA is a crucial determinant of the ability of *C. jejuni* to grow in the cecum. These results are surprising in view of the potentially diverse types of alternative carbon, nitrogen, or energy sources that would be present in this environment and indicate a high degree of selectivity in the types of amino acid utilized. An explanation is provided by the finding that aspartate, glutamate, serine, and proline are in fact the most abundant amino acids present in chicken excreta (25) and that these are the only amino acids (along with asparagine and glutamine) for which *C. jejuni* has the necessary catabolic enzymes.

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