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Elimination of the Vitamin B_{12} Uptake or Synthesis Pathway Does Not Diminish the Virulence of *Escherichia coli* K1 or *Salmonella typhimurium* in Three Model Systems

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The role of iron in infection is of great importance and is well understood. During infection, both the host and the pathogen go through many complicated changes to regulate iron levels. Iron and vitamin B_{12} share certain features. For example, *Escherichia coli* has similar transport systems for both nutrients, and binding proteins for both are located in gastric juice, liver, saliva, granulocytes, and milk. It is because of such parallels between iron and B_{12} that we have explored the role of B_{12} in virulence. A *btuB*::Tn*10* insertion which disrupts the gene encoding the vitamin B_{12} receptor from *E. coli* K-12 was P1 transduced into a virulent *E. coli* K1 strain. In both an infant-rat model and a chicken embryo model, no difference in virulence between the wild-type and the mutant strains was found. Strains of *Salmonella typhimurium* with mutations in the cobalamin synthesis pathway (Cob) and in *btuB* were used in a mouse model of virulence. Mutation of the Cob locus or of *btuB* does not decrease virulence. Interestingly, the inability to synthesize vitamin B_{12} actually increases virulence compared with the wild type in the *S. typhimurium* model. This effect is independent of the B_{12} intake of the mice.

There has long been an interest in nutritional factors and their effect on infection. For example, the role of iron is well understood. During infection there is decreased intestinal absorption of exogenous iron and increased synthesis and stationing of host iron-binding proteins at potential sites of invasion. In addition, organisms generally can acquire iron from the host by using siderophores (iron transport cofactors) (25). There are numerous parallels between iron and vitamin B_{12}. B_{12}-binding proteins are present in gastric juice, liver, saliva, and granulocytes, as are iron-binding proteins (18). There are iron- and B_{12}-binding proteins in milk, with postulated importance in influencing the neonatal gut flora (8). The transport systems for both in *Escherichia coli* have similarities, including the requirement for TonB (2) and significant amino acid homology between the B_{12} receptor and several siderophore-binding proteins (11, 16, 17). These parallels between iron and B_{12} suggested a possible role for this vitamin in virulence.

Salmonella typhimurium synthesizes B_{12} only under anaerobic conditions and requires at least 30 enzymes encoded by the Cob locus to do so (15). *E. coli* is thought to be unable to synthesize this vitamin (19a). Typically, bacteria use B_{12} from the environment. The first stage of transport is the energy-independent binding to the outer membrane protein BtuB, which also serves as the receptor for bacteriophage BF23 and the E and A colicins (7). Active transport into the periplasmic space by BtuB requires the proton motive force and TonB (2). Finally, transport of B_{12} across the cytoplasmic membrane requires the membrane-associated BtuC and BtuD proteins (6).

In *E. coli*, B_{12} is the cofactor for the enzymes ethanolamine ammonia lyase (21) and homocysteine methyltransferase (product of *metH*) (4, 5). The product of *metE* is also a homocysteine methyltransferase but is 57 times slower and does not require B_{12} (10, 23). Vitamin B_{12} is also required to form the modified base queuine present in the anticodon loop of some tRNAs (10). This requirement is not essential under usual growth conditions. In *S. typhimurium* and *Klebsiella* spp., vitamin B_{12} is also required for propanediol dehydratase (13, 24).

The extensive genetic analysis of the B_{12} systems in bacteria has enabled us to address directly the possible role of this nutrient in virulence. In this study, we used previously defined mutations in the uptake pathway of *E. coli* and in the uptake and synthesis pathways of *S. typhimurium* to study their effects in three animal models.

MATERIALS AND METHODS

Media and chemicals. All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. L medium, M63 medium, and MacConkey agar were prepared by the method of Silhavy et al. (22). Colicin E3 was prepared by the method of Foulds and Barrett (9). [^{57}Co]cyanocobalamin and [α-^{32}P]dCTP were purchased from Amersham Corp. (Arlington Heights, Ill.). Vitamin B_{12} levels in mouse blood were determined by Vitamin Diagnostics (Cliffwood Beach, N.J.) by microbiological assay.

Bacterial strains. All strains are described in Table 1. RK4936 from the strain collection of R. Kadner (University of Virginia) was kindly provided by M. Russel. This strain carries a *btuB*::Tn*10* insertion which was P1 transduced into RS218 (O18ac:K1:H7), a virulent P1-sensitive *E. coli* strain isolated in a case of neonatal meningitis (1). Four tetracycline-resistant transductants were shown to lack the B_{12} receptor. They are insensitive to colicin E3 and do not take up [^{57}Co]cyanocobalamin. Southern hybridization studies of one transductant, BAS5010, confirmed the presence of the *btuB* insertion (data not shown). *S. typhimurium* strains were from the laboratory collection of J. Roth. TT16729 (*ara-9 metE1077*) is wild type for the Cob region. *metE1077*

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TABLE 1. Bacterial strains

Strain	Genotype or characteristic	Source
E. coli K-12 derivative RK4936	F⁻ *araD139 ΔargF-lac-205 flbB5301 non-9 gyrA219 relA1 rpsL150 metE70 btuB*::Tn*10 thi-1 deoC1* λ⁻	Laboratory collection of R. Kadner
E. coli K1 derivatives		
RS218	O18ac:K1:H7	Strain 101 (1)
BAS5010	RS218 *btuB*::Tn*10*	This study
S. typhimurium		
TT16729	*ara-9 metE1077*	Laboratory collection of J. Roth
TT16667	*ara-9 metE1077 cob-266*	Laboratory collection of J. Roth
TT16830	*ara-9 metE1077 btuB*::Tn*10*dCam	Laboratory collection of J. Roth
TT16831	*ara-9 metE1077 btuB*::Tn*10*dCam *cob-266*	Laboratory collection of J. Roth
TT16848	*ara-9 metE1077 cobI*::Tn*9*	Laboratory collection of J. Roth
TT16849	*ara-9 metE1077 cob-61*::MudJ	Laboratory collection of J. Roth

is a deletion in *metE*. This allows scoring of cobalamin synthesis, since anaerobic growth of a *metE* strain on minimal medium requires the ability to synthesize B_{12} in order to synthesize methionine (15). TT16667 carries a deletion in Cob called *cob-266*, which removes a large part of the CobI and CobII regions. TT16729 was constructed by correcting this deletion by P22 transduction of the wild-type Cob region from the parent of the strain in which the deletion was originally created. Therefore, these two strains are as isogenic as possible. TT16848 and TT16849 are isogenic with TT16729, except that they carry, respectively, *cobI*::Tn*9* (Tn*9* inserted into the first gene in the CobI region, which is polar on a number of CobI genes but does not affect function of CobII and CobIII) and *cob-61*::MudJ (MudJ inserted near the end of the CobII region, which is polar on very few genes needed for cobalamin synthesis). TT16830 is also isogenic with TT16729 and carries a *btuB*::Tn*10*dCam insertion. TT16848, TT16849, and TT16830 were constructed by P22 transduction of the relevant transposon into TT16729. TT16831 was constructed by P22 transduction of *btuB*::Tn*10*dCam into TT16667 and, therefore, it carries the insertion and the *cob-266* deletion.

Radioactive-B_{12} uptake assay. Overnight cultures of the bacteria to be tested were used undiluted and diluted in L broth to 10^{-1} and 10^{-2}. To 1 ml of water, 150 μl of bacteria and 150 μl of [^{57}Co]cyanocobalamin were added, and the mixture was vortexed and incubated for 30 min at room temperature. A 400-μl aliquot was centrifuged at 10,000 × *g* and washed once, and the ^{57}Co remaining in the pellet was counted in a gamma counter.

Southern hybridization. Chromosomal DNA was isolated and Southern hybridization was performed as described elsewhere (20).

Embryonic-chicken model of bacterial virulence. The embryonic-chicken model of bacterial virulence was recently described by Weiser and Gotschlich (26). Fertilized, antibiotic-free, White Leghorn chicken eggs (SPAFAS, Storrs, Conn.) were housed in a humidified self-turning incubator at 38°C. On day 5, a hole (1 by 1 cm) was made in each shell and covered with tape. After 10 days of incubation, the tape was removed and 50 μl of phosphate-buffered saline (PBS)-washed mid-log-phase *E. coli*, containing the desired concentration of organisms, was applied to the chorioallantoic membrane. Eggs were monitored daily for death.

Neonatal-rat model for *E. coli*. The neonatal-rat model for *E. coli* was described by Bloch et al. (3) and also by Weiser and Gotschlich (26). Timed-pregnant Sprague-Dawley rats were ordered from Taconic Farms, Germantown, N.Y. Within 24 h of birth, infant rats were randomized and given either 10 μl orally (p.o.) or 100 μl intraperitoneally (i.p.) of PBS-washed, mid-log-phase organisms of the desired concentration. Animals which died and which had positive spleen cultures were considered to have died from overwhelming bacteremia. All pups were sacrificed 72 h postinfection, and 100 μl of blood obtained by percutaneous cardiac puncture was cultured.

Mouse model for *S. typhimurium*. Six- to eight-week-old male BALB/c or C3H mice (Charles River Laboratories, Wilmington, Mass.) were inoculated with 10 μl p.o. or 100 μl i.p. of PBS-washed, mid-log-phase organisms of the desired concentration. Mice were monitored twice daily for 30 days, and morbid animals were euthanized. Autopsies of the first 200 mice which died demonstrated characteristic splenomegaly, and *S. typhimurium* could be cultured from their spleens. *S. typhimurium* could be cultured from the stools of all infected mice by 1 week postinfection. The mice which survived to day 30 cleared the infection, as no *S. typhimurium* organisms were cultured from the spleen. In certain experiments, cyanocobalamin (American Reagent Corp. Shirley, N.Y.) was administered daily by subcutaneous injection (0.025 μg) and by water supplementation (~1 mg). Control animals were injected with sterile saline.

RESULTS

A *btuB* insertion has no effect on the virulence of *E. coli* K1. A *btuB*::Tn*10* insertion was introduced into the *E. coli* K1 strain RS218 by P1 transduction, resulting in BAS5010. Since it lacks the B_{12} receptor, this strain is insensitive to colicin E3 and takes up no [^{57}Co]cyanocobalamin. Furthermore, the strain was confirmed by Southern hybridization to have a *btuB* insertion (data not shown). This mutant and the wild-type RS218 have identical growth rates in rich media (data not shown). These two strains were then tested in the two animal systems.

In the chicken embryo model, the inoculum is placed on the chorioallantoic membrane at day 10 of gestation. Death is monitored by candling each egg for 3 days postinfection. Weiser and Gotschlich (26) showed that a few organisms are sufficient to kill the embryo, usually by the second day of the infection. Sham inoculation of sterile PBS resulted in a mortality rate of about 3%, most likely due to handling of the delicate embryos.

As shown in Table 2, at a low dose RS218 killed 37.5% of

TABLE 2. Lethality of the *btuB*::Tn*10* *E. coli* K1 mutant in the chicken embryo model

Strain	Inoculum (no. of bacteria)	No. dead/no. inoculated (% dead)
RS218	2.5×10^3	14/16 (87.5)
BAS5010 (*btuB*::Tn*10*)	1.0×10^3	21/22 (95.5)
RS218	250	6/16 (37.5)
BAS5010 (*btuB*::Tn*10*)	105	5/20 (25)

the embryos, while BAS5010 (*btuB*::Tn*10*) killed 25%. This difference is not significant ($P > 0.1$ as determined by chi-square analysis). Similarly, at a high dose, RS218 caused a mortality rate of 87.5% and BAS5010 caused a mortality rate of 95.5%. Again, the difference between strains is not significant ($P > 0.1$).

Bloch et al. (3) have developed a neonatal-rat model in which RS218 causes bacteremia and death. We have found that results from i.p. inoculation are less reliable than those from p.o. inoculation because the infant rats are so susceptible to the organisms at this early age. Results from both p.o. and i.p. inoculations are shown in Table 3. p.o. inoculations of RS218 and BAS5010 killed 64 and 54% of the animals, respectively ($P > 0.1$), while i.p. inoculations killed 45.5 and 80%, respectively ($P > 0.1$). The results from both the chicken embryo and the neonatal-rat models are consistent, and they show that the *btuB*::Tn*10* insertion has no effect on the virulence of *E. coli* K1 in these systems.

Inability to synthesize vitamin B_{12} in *S. typhimurium* increases virulence. We used five mutants of *S. typhimurium* in the mouse model of virulence (Table 1). The first strain, TT16667, has a large deletion in the cobalamin synthesis genes which removes the CobI and CobII loci. It is important that the wild-type strain used was constructed by repairing this deletion of the Cob region by P22 transduction. Hence, these strains are as isogenic as possible. The second mutant, TT16830, has an insertion in the *btuB* gene (*btuB*::Tn*10*dCam). The third strain, TT16831, is a double mutant carrying both the described *cob-266* and *btuB* mutations in the same strain. The fourth and fifth strains, TT16848 and TT16849, have different insertions in the Cob region, which both abolish B_{12} synthesis but at two different points in the pathway. The two insertions are polar on different sets of genes. Growth rates of all strains in rich media were identical (data not shown).

Figure 1 shows the rates of death of mice infected with the wild type, the Cob deletion mutant, the *btuB* insertion mutant, or the Cob deletion-*btuB* insertion double mutant. The time course of death represented here is typical of experiments in this study with the same strain and inoculum. With the more virulent strains, half the deaths have occurred by day 15 postinfection. Although no mice died upon i.p.

TABLE 3. Lethality of the *btuB*::Tn*10* *E. coli* K1 mutant in the neonatal-rat model

Strain	No. of bacteria (route of inoculation)	No. dead or bacteremic/ no. inoculated (% dead)
RS218	5×10^8 (p.o.)	16/25 (64)
BAS5010 (*btuB*::Tn*10*)	5×10^8 (p.o.)	14/26 (54)
RS218	41 (i.p.)	5/11 (45.5)
BAS5010 (*btuB*::Tn*10*)	56 (i.p.)	8/10 (80)

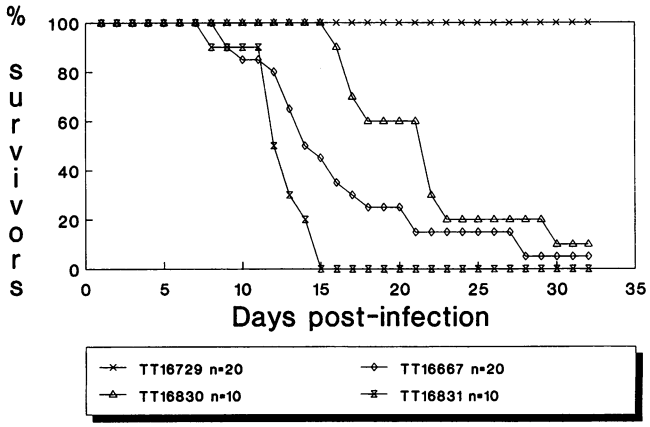

FIG. 1. Comparison of the percent survivors versus time postinfection with TT16729, TT16667 (*cob-266*), TT16830 (*btuB*::Tn*10*dCam), and TT16831 (*btuB*::Tn*10*dCam *cob-266*). n, number of mice in each experimental group. BALB/c mice were infected i.p. with approximately 100 bacteria.

injection of wild-type organisms, 95% died after receiving an equal number of the organisms carrying the *cob-266* mutation ($P < 0.001$). In this experiment the *btuB* mutant, which caused a 90% mortality rate, was also more virulent than the wild type but not as virulent as the *cob-266* mutant ($P < 0.001$). The time course of death reflects this lesser virulence, as half the deaths did not occur until day 22 postinfection. The *cob-266 btuB* mutant displays the same greater virulence as the strain containing only the *cob-266* mutation ($P < 0.001$), killing 100% of the mice infected. Table 4 shows, in addition to these data, that two other Cob mutants behaved as did the mutant with the *cob-266* deletion. TT16848 and TT16849, strains with different insertions in different parts of the Cob region with polarity effects on separate genes, killed 100% of the mice infected. When the i.p. inoculum was increased to 10^4 bacteria, all infected mice died irrespective of the strain used.

Similar results are obtained when mice are inoculated p.o. While none of the 20 mice infected with TT16729 died, 12 of 20 mice infected with TT16667 died ($P < 0.001$). TT16831 killed 4 of 10 mice ($P < 0.001$). However, the *btuB* insertion mutant (TT16830) is significantly less virulent than the Cob deletion in this model. It killed only 1 of 10 mice ($P > 0.1$). This result, together with the delay in the onset of death when mice were infected i.p. with this strain, suggests that although it is more virulent than wild type, the *btuB* insertion mutant is not as virulent as the mutants unable to synthesize vitamin B_{12}.

Furthermore, when C3H mice, which are normally more

TABLE 4. Lethalities of defined *S. typhimurium* strains with mutations in vitamin B_{12} synthesis or uptake for BALB/c mice

Strain	No. dead/no. inoculated i.p. with 100 bacteria (% dead)	No. dead/no. inoculated p.o. with 10^8 bacteria (% dead)
TT16729	0/20 (0)	0/20 (0)
TT16667	19/20 (95)	12/20 (60)
TT16848	10/10 (100)	ND[a]
TT16849	10/10 (100)	ND
TT16830	9/10 (90)	1/10 (10)
TT16831	18/18 (100)	4/10 (40)

[a] ND, not determined.

TABLE 5. Effect of vitamin B_{12} administration on *S. typhimurium* mutants in the BALB/c mouse model

Strain	No. dead/no. inoculated i.p. with 100 bacteria (% dead)	
	Without B_{12}	With B_{12}
TT16729	0/10 (0)	0/10 (0)
TT16667	12/18 (66)	17/19 (89)
TT16831	8/8 (100)	8/8 (100)

resistant to *S. typhimurium* infection (19), were used instead of BALB/c mice, the same pattern of virulence again emerged. At an inoculum of 10^3 bacteria, none of the five mice infected with TT16729 died, while four of the six infected with TT16667 died ($P < 0.001$).

Table 5 shows the effect of administering vitamin B_{12} by daily subcutaneous injection (0.025 μg) with water supplementation (~1 mg) on virulence. Control mice received daily subcutaneous injections of saline. Examination of representative B_{12} levels in the blood confirmed that B_{12} levels rose from 11,500 to 40,000 pg/ml (Vitamin Diagnostics). Addition of B_{12} neither significantly increased nor significantly decreased the virulence of the wild type, the *cob-266* deletion mutant, or the *cob-266* deletion–*btuB* insertion double mutant. B_{12} administration also had no effect on the virulence of the mutant containing only the *btuB* insertion (data not shown).

DISCUSSION

Since the isolation of vitamin B_{12} in 1948, scientists and clinicians alike have been fascinated by this corrin ring surrounding a cobalt atom (12). The uptake of vitamin B_{12} by bacteria has been studied for over 20 years, particularly, although not exclusively, with *E. coli* and with *S. typhimurium*. The resulting literature reveals a complex system which shares many aspects with the uptake of iron. Both have outer membrane receptor proteins which serve as colicin and phage receptors as well. They each require the TonB protein to serve as an energy coupler (2).

The anaerobic synthesis of vitamin B_{12} in *S. typhimurium* has been extensively characterized (14). It requires an extensive array of genes, occupying approximately 1% of the genome (15, 19a). Although B_{12} has been shown to be important as a cofactor for several reactions, such as homocysteine methyltransferase (4, 5) and ethanolamine ammonia lyase (21) reactions and propanediol utilization (13, 24), no crucial function has yet been assigned to this molecule, which the organism goes to great lengths to take up or to synthesize (14).

The role of iron in infection has been firmly established (25). The host withholds iron from the bacterium. The bacterium, in turn, has evolved the means by which to take iron from the host, most commonly using siderophores to bind iron from the environment or to "steal" it from the host binding proteins. The parallels between B_{12} and iron were suggestive of a role for B_{12} in virulence. Even more suggestive was the presence of iron- and B_{12}-binding proteins in milk, granulocytes, gastric juice, saliva, and the liver (8, 18). Speculation about the possible antimicrobial properties of these B_{12}-binding proteins is present in the literature, although to our knowledge no definitive studies have been published.

We took advantage of the extensive genetic study of the B_{12} uptake system to introduce defined mutations into two virulent bacterial species, *E. coli* K1 and *S. typhimurium*. Since *S. typhimurium* is capable of synthesizing B_{12} anaerobically, we also studied the effects of synthesis mutants in this species. Recently, Weiser and Gotschlich (26) published a study utilizing RS218, a P1-sensitive virulent *E. coli* K1 strain in the embryonic-chicken model of virulence. They found this model to be a reliable indicator of the relative virulence of the mutants tested. Bloch et al. (3) and Weiser and Gotschlich (26) used the same strain in studies using an infant-rat model for infection, and again this strain proved useful. The mouse model for the study of *S. typhimurium* virulence is very well worked out. It involves a natural infection and mimics even the route of infection when bacteria are inoculated p.o.

We have shown in this study that the absence of BtuB has no effect on the virulence of *E. coli* K1 in the embryonic-chicken and neonatal-rat models. *S. typhimurium* TT16729 was constructed by correcting the *cob-266* deletion in TT16667 by P22 transduction. Therefore, these two strains are as isogenic as possible, making comparison of their virulence levels particularly relevant and revealing. In several separate experiments using the mouse model for virulence of *S. typhimurium*, the Cob mutant is more virulent than the wild type. The decrease in 50% lethal dose is approximately 1 to 2 orders of magnitude. Similarly, two insertion mutants which are isogenic with TT16729 and which eliminate cobalamin synthesis were also more virulent than the wild type. The *btuB* mutant is also more virulent than the wild type but not as virulent as the Cob mutant. The *cob-266 btuB* double mutant acts like the strain containing only the *cob-266* mutation. This pattern of virulence is not affected by increasing the internal B_{12} level of the mice.

This study suggests that the role of vitamin B_{12} in bacterial pathogenesis is very different from that of iron. Inability to synthesize or to transport B_{12} does not cripple the organism. To the contrary, it increases virulence in the *S. typhimurium* model. It is rare that a mutation increases virulence. It is possible that vitamin B_{12} within the bacterium is capable of regulating a gene or a group of genes which directly or indirectly control a virulence factor. Another possibility is that the vitamin B_{12} produced by the bacterium is somehow sensed by the host. This detection might conceivably cause an altered response by the host that results in a less virulent infection. With so much genetic information dedicated to the uptake and synthesis of vitamin B_{12} and no essential role for it yet defined, it is obvious that there is still much to learn about the role of B_{12} in bacteria.

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