

# Activity Spectrum of Colicins Produced by *Shigella sonnei* and Genetic Mechanism of Colicin Resistance in Conspecific *S. sonnei* Strains and *Escherichia coli*

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Colicin-mediated killing is an example of allelopathy, which has been found among several bacteria. Screening of 42 strains of *Shigella sonnei* isolated from diarrheal patients revealed that 39 (93%) *S. sonnei* strains were positive for colicin production against *Escherichia coli* DH5 $\alpha$ . In the PCR-based detection of the colicin types, 36 (92.3%) were identified as E3, 2 (5.1%) as E3 and E8, and 1 (2.6%) as E3 and E2. Representative *S. sonnei* strains producing heterologous colicins exhibited antagonism against diarrheagenic *Escherichia coli* (DEC) groups. Although it is known that mutation in the colicin receptor renders the host resistant to colicin, there is a dearth of information on the genetic characterization of such mutants. In the fluctuation test, colicin-resistant *E. coli* mutants were found to occur spontaneously at the rates of  $2.51 \times 10^{-8}$  and  $5.52 \times 10^{-8}$  per generation when exposed to colicins E3 and E8 and colicins E3 and E2, respectively. Genotypic characterization of colicin-resistant *E. coli* (EC<sup>Cr</sup>) and *S. sonnei* (SS<sup>Cr</sup>) strains displayed mutations in the *btuB* gene, which encodes the receptor for vitamin B<sub>12</sub> uptake. This gene was interrupted by various insertion sequences, such as IS1, IS2, and IS911. Complementation of EC<sup>Cr</sup> and SS<sup>Cr</sup> with plasmid-borne *btuB* (*pbtuB*) accomplished restoration of the colicin-susceptible phenotype. The vitamin B<sub>12</sub> uptake assay gave an insight into the physiological relevance of the *btuB* mutation. Our studies provide insights into the latent influence of *S. sonnei* colicins in governing the existence of some of the shigellae and all of the DEC and the genetic mechanism underlying the emergence of resistance.

Allelopathy refers to the production of toxic metabolites that suppress both the growth and survival of distinct competitors in a common niche (1, 2). Colicin is one such antimicrobial biomolecule produced by certain members of the family *Enterobacteriaceae*, which provides a competitive edge against microorganisms that are not immune or resistant (3–6). Colicin producers express the immunity protein constitutively, which forms a complex with the colicin protein, thereby preventing “cell suicide.” Resistance to killing is attributable to two factors: (i) a mutation in the receptor, which serves as a portal for entry into the target cell (true resistance), and (ii) an alteration in some component, which constitutes a part of the translocation machinery (tolerance) (7, 8).

In the present study, we have focused primarily on the role of colicin produced by the *Shigella sonnei* strains isolated from acute diarrheal patients in preventing the growth of diarrheagenic *Escherichia coli* (DEC) groups and spontaneous emergence of resistance to colicin due to mutation in the *btuB* gene, which encodes the receptor for vitamin B<sub>12</sub> uptake. The BtuB protein also serves to localize type A/E colicins and T5-like phages (BF23 and EPS7) on the target cell surface (9, 10). The *btuB* gene consists of a single open reading frame, which is translated into a 614-amino-acid polypeptide. The first 20 amino acids constitute a signal peptide, which gets cleaved during secretion across the cytoplasmic membrane. The remaining 594 amino acid residues yield the mature/processed protein, which has a molecular mass of 66 kDa (11, 12). All of the BtuB molecules are capable of transporting vitamin B<sub>12</sub> and also facilitate the killing effect of phage BF23, but only a proportion of the receptors, which are newly synthesized, can mediate colicin action (12).

There is a wealth of information on colicin production by *E. coli*. However, there are very few reports on the frequency and types of colicins produced by *S. sonnei* as well as their role in

governing the population dynamics of other gut pathogens, especially in the case of polymicrobial infections. *S. sonnei* is the causative agent of acute bacillary dysentery, which has a low infectious dose ranging from 10 to 100 live cells. Of the four serogroups (*S. sonnei*, *S. boydii*, *S. flexneri*, and *S. dysenteriae*), *S. sonnei* is an exception because it is not further subdivided into subgroups, and hence, in the past colicin production was used as an additional epidemiological marker for typing *S. sonnei* strains (13, 14). Among the colicins produced by *Shigella* strains, colicin U and colicin Js are expressed by *S. boydii* and *S. sonnei*, respectively (15–17). A few studies have demonstrated that *S. sonnei* colicins exhibit antagonism against some of the shigellae and DEC, which are sometimes uncontrollable by any antimicrobial therapy (18). Hence, strategies for designing newer antibiotics with different modes of action are now being considered. Contrary to the indiscriminate killing approach of broad-spectrum antibiotics, colicins are highly targeted in their action, as they only cripple the target pathogen without causing collateral damage to the commensal

Received 25 August 2014 Returned for modification 26 September 2014

Accepted 1 October 2014

Accepted manuscript posted online 20 October 2014

Citation Calcuttawala F, Hariharan C, Pazhani GP, Ghosh S, Ramamurthy T. 2015. Activity spectrum of colicins produced by *Shigella sonnei* and genetic mechanism of colicin resistance in conspecific *S. sonnei* strains and *Escherichia coli*. *Antimicrob Agents Chemother* 59:152–158. doi:10.1128/AAC.04122-14.

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.04122-14>.

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TABLE 1 Primers used for sequencing the entire *btuB* gene

Target	Primer(s) (5' to 3')	PCR conditions	Amplicon size (bp)	Reference or source
BtuB-P	CGGGGTACCGATACCAGCCCGGAT and CCCAAGCTTTT CAGAAGGTGTAGCT	94°C for 1 min, 57°C for 1 min, 72°C for 1 min 30 s, 30 cycles	1,746	44
BtuB-D	GGATCCATGATTA AAAAAGCTTCGCTG and GGATCCTCAGAAGGTGTAGCTGCCAG	94°C for 1 min, 57°C for 1 min, 72°C for 1 min 30 s, 30 cycles	1,859	This study
BtuB-1	CTGCTGACGGCGTGTTCCTG and CGCCCAACAGCGTTACCCGT	94°C for 30 s, 63°C for 45 s, 72°C for 45 s, 30 cycles	553	This study
BtuB-2	ACGGGTAACGCTGTTGGGCG and CACTGCGTCTGGCGCCTTCA	94°C for 30 s, 63°C for 45 s, 72°C for 45 s, 30 cycles	587	This study
BtuB-3	TGAAGGCGCCAGACGCAGTG and CGACGCCCTTAATCCGCGCT	94°C for 30 s, 63°C for 45 s, 72°C for 45 s, 30 cycles	335	This study
BtuB-4	TCACGACGCGGATGAACCC and GTTATAGCGCAGCCCGGCGT	94°C for 30 s, 63°C for 45 s, 72°C for 45 s, 30 cycles	383	This study
BtuB3RF2	AGTGGGAAGGCGCGTTTGAAG	Used directly for sequencing		This study
BtuB3RR2	GCAGAGGCGTTCGAGCATTAT	Used directly for sequencing		This study
IS2	GCCGCCGAGTCGTACAC and CCATTATCCGTCAGCCACTCCACT	94°C for 45 s, 57°C for 45 s, 72°C for 45 s, 30 cycles	478	This study
IS911	CCTGCTGTCGGCGGAGAACG and CAAAAGCCTCTCGGATAACACCAG	94°C for 30 s, 70°C for 30 s, 72°C for 30 s, 25 cycles	627	This study
BtuB-5F	ACGGTAGTATTGGTGGCGGTGTCG and IS1-R2	94°C for 30 s, 70°C for 30 s, 72°C for 30 s, 25 cycles	481	This study
BtuB-6	GATGCAATAGGCGGGGTGGTGA and CCGCTCCAGGCATCAGTAAAGTT	94°C for 30 s, 70°C for 30 s, 72°C for 30 s, 25 cycles	286	This study

bacteria (19). As colicins target a narrow phylogenetic range of microorganisms, the selection of mutations that confer resistance to the toxin will take place only in a small fraction of the microbial community instead of multiple species simultaneously. This study provides a unique insight into the action of *S. sonnei* colicins and the genetic basis of their resistance.

## MATERIALS AND METHODS

**Bacterial strains.** Forty-two strains of *S. sonnei* isolated from stool specimens of acute diarrheal patients admitted to the Infectious Diseases Hospital (IDH), Kolkata, India, were screened for colicin production using *E. coli* DH5 $\alpha$  (colicin-sensitive *E. coli* [EC<sup>CS</sup>]) as an indicator strain. In the active surveillance, every 5th patient admitted on any two randomly selected days in a week was enrolled in the study. Three representative *S. sonnei* strains (IDH01791, IDH01157, and 500867) with heterologous colicin types (selected on the basis of PCR screening [20] and cross-immunity testing) were used for detecting their antagonistic activity against commensal *E. coli* strains that did not harbor any virulence genes, enterotoxigenic (ETEC), typical enteropathogenic (tEPEC), atypical enteropathogenic (aEPEC), enterohemorrhagic *E. coli* (EHEC), and enteroaggregative (EAEC) *E. coli*, along with *S. dysenteriae* type 1, *S. flexneri* 2a, and *S. sonnei*. Cloning and transformation were performed using the Mach1-T1<sup>r</sup> strain [F<sup>-</sup>  $\Phi$ 80lacZ $\Delta$ M15  $\Delta$ lacX74 *hsdR*(r<sub>k</sub><sup>-</sup> m<sub>k</sub><sup>+</sup>)  $\Delta$ recA1398 *endA1 tonA*] (Invitrogen, Carlsbad, CA).

**Colicin assay.** For studying colicin production, a single colony of one of the previously confirmed strains of *S. sonnei* from xylose lysine desoxycholate agar (Difco, Sparks, MD, USA) was inoculated into 5 ml of Luria-Bertani (LB) broth (Difco) and incubated overnight at 37°C in a shaker. On the following day, the culture was centrifuged at 10,000  $\times$  g for 10 min. The culture supernatant from each strain was passed through a 0.22- $\mu$ m filter (Millipore, Bangalore, India). About 10  $\mu$ l of the crude filtrate was spotted on the Mueller-Hinton agar (MHA) (Difco) plate, previously inoculated with the log phase culture of *E. coli* DH5 $\alpha$  or the other test strains. Plates were incubated at 37°C overnight and checked for the presence or absence of a zone of inhibition on the following day.

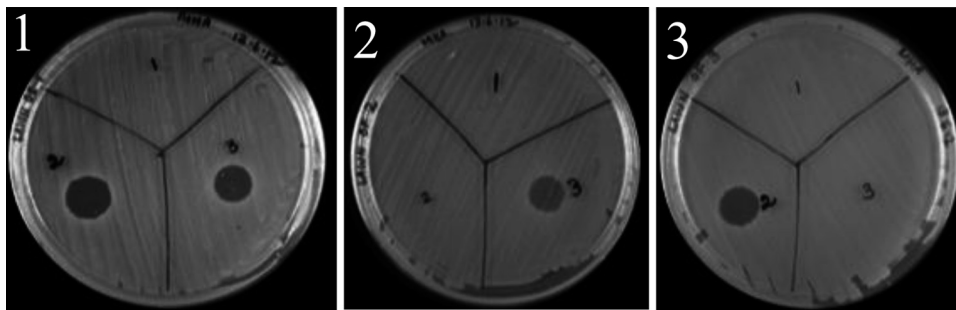
**Isolation of colicin-resistant mutants.** In the colicin assay, a few colicin-resistant colonies were found to grow as suppressed colonies within

the zone of inhibition on the MHA plate seeded either with *E. coli* DH5 $\alpha$  or *S. sonnei*. Some of these colicin-resistant colonies of *E. coli* (EC<sup>Cr</sup>) DH5 $\alpha$  and *S. sonnei* (SS<sup>Cr</sup>) were selected for further investigation. The colicin-resistant colonies were confirmed as *E. coli* and *S. sonnei* using biochemical tests and serological testing, respectively. In addition, pulsed-field gel electrophoresis (PFGE) was performed (21) to confirm their DNA fingerprints by comparing the colicin-susceptible progenitor and colicin-resistant strains. To detect the differences, if any, in the sugar fermentation abilities of the resistant and sensitive strains, an API 32E (bioMérieux, Marcy l'Étoile, France) test was performed according to the manufacturer's protocol.

**Amplification and sequencing of the *btuB* gene.** The *btuB* gene of EC<sup>Cr</sup>, SS<sup>Cr</sup>, and their colicin-susceptible progenitors was amplified using the BtuB-P primer pair (Table 1). Amplicons were sized against molecular weight markers by agarose gel electrophoresis and purified using a PCR product purification kit (Qiagen, Hilden, Germany). The amplicons were sequenced with forward and reverse primers using an automated DNA sequencer (ABI 3730; Applied Biosystems, Foster City, CA). To sequence the larger amplicons of the resistant mutants, several internal primers were used (Table 1). The sequences were assembled and analyzed using DNASTAR software (DNASTAR, Inc., Madison, WI).

**Genetic complementation of EC<sup>Cr</sup> and SS<sup>Cr</sup> with intact *btuB*.** The complete *btuB* gene was amplified using the primer pair BtuB-D and the template DNA from an EC<sup>CS</sup> colony. The PCR product was used for Topo TA cloning (Invitrogen). One Shot Mach1-T1<sup>r</sup> competent cells (Invitrogen) were chemically transformed using the recommended protocol. The transformants were selected on LB agar plates containing 30  $\mu$ g/ml of kanamycin. A PCR assay was performed using the M13 primers to confirm the *btuB* gene in the transformants. A single colony of the *btuB*-harboring clone was subjected to plasmid DNA isolation. Chemically competent EC<sup>Cr</sup> and SS<sup>Cr</sup> were transformed with the plasmid DNA (*pbtuB*). The selection was done on LB agar with kanamycin (30  $\mu$ g/ml) plates. A colicin assay was performed to check for restoration of a colicin-susceptible phenotype (22).

**Uptake of vitamin B<sub>12</sub> in the colicin-susceptible, -resistant, and -complemented strains of *E. coli* DH5 $\alpha$  and *S. sonnei*.** The uptake of vitamin B<sub>12</sub> was quantified using the vitamin B<sub>12</sub> assay medium (Difco). A



**FIG 1** Antagonism between colicin-producing *S. sonnei* using the crude filter-sterilized supernatant from IDH01791 (1), IDH01157 (2), and 500867 (3) on a lawn culture of each of the aforementioned strains.

stock concentration of vitamin B<sub>12</sub> (400 µg/ml) (SRL, Mumbai, India) was prepared using 25% ethanol (Merck, Mumbai, India). A *Lactobacillus leichmannii* (ATCC 7830) strain, which is a vitamin B<sub>12</sub>-dependent auxotroph, was used as a test organism (23). After an initial standard curve using the test organism was obtained, different concentrations of vitamin B<sub>12</sub> (80, 120, and 160 pg) were designated for the assay. Colicin-susceptible, -resistant, and -complemented strains of *E. coli* DH5α and *S. sonnei* were inoculated into normal LB broth (LBN) containing 800, 1,200, and 1,600 pg of vitamin B<sub>12</sub>, and after overnight incubation at 37°C in a shaker, the cells were centrifuged at 10,000 × *g* for 10 min. The culture supernatant was sterilized by passage through a 0.22-µm filter (Millipore). To 5 ml of double-strength vitamin B<sub>12</sub> assay medium, 1 ml of the crude filtrate was added, and the volume was made up to 10 ml with sterile distilled water. In the case of controls, instead of the crude filtrate, 200, 300, and 400 µl of vitamin B<sub>12</sub> (400 pg/ml) was added. Uninoculated and inoculated controls (without vitamin B<sub>12</sub>) were also included in the assay. After autoclaving of the above medium at 121°C for 5 min, all of the test tubes (except the uninoculated control) were inoculated with *L. leichmannii* and incubated overnight at 37°C in a static condition. The optical density at 530 nm (OD<sub>530</sub>) was recorded on the following day, and a graph of the OD<sub>530</sub> versus the amount of vitamin B<sub>12</sub> (pg) was plotted in order to compare the vitamin B<sub>12</sub> uptake of colicin-sensitive, -resistant, and -complemented strains of *E. coli* and *S. sonnei* (24–26).

**Luria-Delbrück fluctuation assay.** The Luria-Delbrück fluctuation assay (27) was adopted with a slight modification to check the average mutation rate. In brief, a series of 10 tubes containing 200 µl of LBN and a single 100-ml conical flask containing 10 ml of LBN were each inoculated with EC<sup>CS</sup> to a concentration of 500 cells/ml. Cells were incubated in a 37°C shaker until they attained an OD<sub>600</sub> of about 1.0. One hundred-microliter aliquots were taken from each of the small cultures, and ten 100-µl aliquots were taken from the 10-ml bulk culture. These samples were spread on normal LB agar plates containing crude colicin extract from the strain IDH01157 or 500867, respectively. The plates were incubated overnight, and on the following day, the number of colonies on each plate was counted. Representative colonies were picked from each plate and subcultured onto colicin-containing plates. Further, their resistance toward colicin produced by IDH01791, IDH01157, and 500867 was confirmed. A PCR was carried out using the BtuB-P primers, and the percentage of mutants arising due to a disruption of the *btuB* gene by the insertion sequence (IS) element was determined. The mutation rates were estimated using the Ma-Sandri-Sarkar maximum likelihood estimator (MSS-MLE) implemented by the FALCOR webtool (<http://www.keshavsingh.org/protocols/FALCOR.html>) (27).

**Nucleotide sequence accession numbers.** The *btuB* gene sequences disrupted by the IS elements in the colicin-resistant mutants were submitted to GenBank. The accession numbers for EC<sup>Cr</sup>A1, EC<sup>Cr</sup>A11, SS<sup>Cr</sup>A2, and SS<sup>Cr</sup>A11 reported in this paper are KJ494659, KC806221, KJ494660, and KC806222, respectively.

## RESULTS

**Detection of colicin-producing *S. sonnei* strains and their antagonistic activity against different enteric bacteria.** Of the 42 strains of *S. sonnei* screened, 39 (93%) were identified as colicin producers based on the phenotypic screening by the colicin assay using *E. coli* DH5α. PCR-based detection of the colicin type was carried out using the subset of 39 producers and previously published primers (20). Thirty-six strains (92.3%) were identified as colicin type E3, 2 (5.1%) as types E3 and E8, and 1 (2.6%) as types E3 and E2. Three representative *S. sonnei* strains, IDH01791 (colicin E3 producer), IDH01157 (producing colicins E3 and E8), and 500867 (producing colicins E3 and E2), with a heterologous colicin profile were selected for further studies. Cross-immunity tests between these colicin producers were performed to substantiate the results obtained by the PCR (Fig. 1). In addition, colicin-mediated antagonistic activity against several gut pathogens was tested. Overall, the colicin susceptibilities of the diarrheagenic *E. coli* (DEC) were as follows: tEPEC, 35% (*n* = 20); aEPEC, 55% (*n* = 20); EAEC, 35% (*n* = 20); ETEC, 55% (*n* = 20); and EHEC, 50% (*n* = 4). Among the *Shigella* spp., 33% of *S. dysenteriae* type 1 (*n* = 12) and 5% of *S. flexneri* 2a (*n* = 22) exhibited colicin susceptibility. The majority of the E3 type of colicin-producing *S. sonnei* strains (97%, *n* = 32) were susceptible to the IDH01157 and 500867 strains, which produce dual colicin types of E3 and E8 and E2 and E3, respectively. We found that a large proportion of the commensal *E. coli* (85%, *n* = 20) that did not harbor any of the DEC virulence genes were resistant to colicin.

**Isolation and characterization of colicin-resistant mutants based on phenotypic markers.** *S. sonnei* colicins exerted their lethal effect against different pathogroups of DEC. In addition, most of the *S. sonnei* strains, which were either heterologous or non-colicin producers, were found to be susceptible. However, in a few strains, the resistant mutants were found to grow as suppressed colonies within the zone of inhibition. Several such mutants were analyzed to gain an insight into the genetic mechanism for colicin resistance. These included colicin-resistant *E. coli* DH5α isolated from the zone of inhibition of the colicins produced by *S. sonnei* strains IDH01791 (EC<sup>Cr</sup>A1 to EC<sup>Cr</sup>A11), IDH01157 (EC<sup>Cr</sup>B1 to EC<sup>Cr</sup>B11), and 500867 (EC<sup>Cr</sup>C1 to EC<sup>Cr</sup>C15). Similarly, colicin-resistant *S. sonnei* IDH01791 isolated from the zone of inhibition of the colicin-producing conspecific *S. sonnei* strains IDH01157 (SS<sup>Cr</sup>A1 to SS<sup>Cr</sup>A11) and 500867 (SS<sup>Cr</sup>B1 to SS<sup>Cr</sup>B16) was also analyzed. The second category of mutants merits special attention as it marks the competition between two

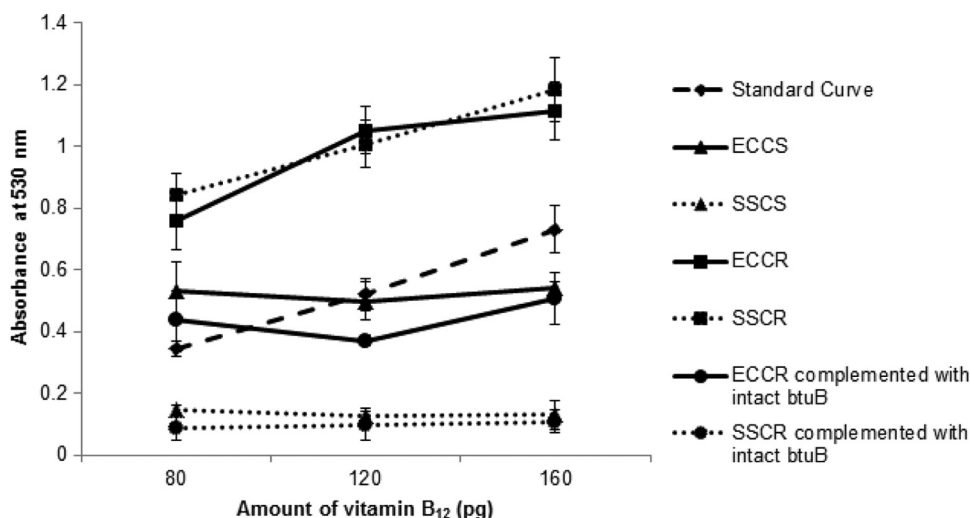


FIG 2 Comparison of vitamin B<sub>12</sub> uptake in EC<sup>Cr</sup>, EC<sup>Cr</sup>, SS<sup>Cr</sup>, and SS<sup>Cr</sup> and the genetically complemented strains. The results are expressed as means and standard deviations from three independent experiments.

conspicuous colicin producers. Representative mutant strains of EC<sup>Cr</sup> and SS<sup>Cr</sup> were confirmed as *E. coli* and *S. sonnei*, respectively, in the biochemical testing and slide agglutination assays using the specific polyclonal antisera. The API 32E test results revealed that there was no change in the biochemical fermentation profiles of the progenitor and resistant mutants (data not shown). In the PFGE, the progenitor and mutant strains were found to be identical (data not shown).

**PCR-based amplification of the *btuB* gene.** The resistant colonies of each category of mutants were subjected to *btuB* PCR assays. Analysis of the PCR products by agarose gel electrophoresis showed fragments of about 3,000 bp from the majority of the EC<sup>Cr</sup> colonies. In the case of SS<sup>Cr</sup>, PCR products of sizes approximating 2,500 bp and 3,000 bp were obtained from most of the colonies. These unexpected large-sized amplicons indicated that the *btuB* gene was interrupted by the insertion of DNA fragments in the resistant mutants but not in the colicin-susceptible progenitors that gave a desired amplicon (1,746 bp).

**Sequence analysis of *btuB* in EC<sup>Cr</sup> and SS<sup>Cr</sup> mutants.** DNA sequencing and BLAST analysis of representative mutants with different amplicon sizes (EC<sup>Cr</sup>A1, EC<sup>Cr</sup>A11, SS<sup>Cr</sup>A2, and SS<sup>Cr</sup>A11) showed insertion sequences (IS elements) interrupting the *btuB* gene. IS1 (~770 bp) and IS2 (~1,300 bp) were detected in EC<sup>Cr</sup>A1 and EC<sup>Cr</sup>A11, respectively, whereas IS911 (~1,250 bp) and IS1 (~770 bp) were found to disrupt the *btuB* gene of SS<sup>Cr</sup>A2 and SS<sup>Cr</sup>A11, respectively. These IS elements differed in their orientation and insertion sites within the *btuB* gene. The IS was incorporated in the same direction as that of *btuB* transcription in EC<sup>Cr</sup>A11, whereas in EC<sup>Cr</sup>A1, SS<sup>Cr</sup>A2, and SS<sup>Cr</sup>A11, the IS was oriented in the reverse direction. With the reference sequence of the *E. coli btuB* gene for the vitamin B<sub>12</sub> receptor protein BtuB (GenBank accession no. M10112), the IS element was identified at positions 1392, 1689, 1176, and 1827 in EC<sup>Cr</sup>A1, EC<sup>Cr</sup>A11, SS<sup>Cr</sup>A2, and SS<sup>Cr</sup>A11, respectively. In the four mutants, the flanking region of the IS element exhibited typical direct repeats.

**Complementation with intact *btuB* restores a colicin-susceptible phenotype.** In the colicin assay, the transformants

SS<sup>Cr</sup>A11*btuB*::IS1 and EC<sup>Cr</sup>A11*btuB*::IS2 complemented with *pbtuB* exhibited susceptibility to colicin, thereby highlighting the role of intact *btuB* in this transfiguration. This confirms that IS elements are the exclusive determinants of colicin resistance in the aforementioned mutants.

**Comparison of vitamin B<sub>12</sub> uptake in colicin-susceptible, -resistant, and -complemented strains of *E. coli* and *S. sonnei*.** The vitamin B<sub>12</sub> uptake assay was performed to determine the influence of the mutation in the *btuB* gene on the vitamin B<sub>12</sub> uptake ability of the organism. In the cases of EC<sup>Cr</sup> and SS<sup>Cr</sup>, a drop in the OD<sub>530</sub> value was recorded compared to those for EC<sup>Cr</sup> and SS<sup>Cr</sup>, due to normal uptake of vitamin B<sub>12</sub> by the colicin-susceptible strains. Much less vitamin B<sub>12</sub> remained in the spent medium that supported the growth of *L. leichmannii* and hence the reduction in the OD<sub>530</sub> value. In the cases of EC<sup>Cr</sup> and SS<sup>Cr</sup>, there was decreased uptake of vitamin B<sub>12</sub> by the mutant, thereby enhancing the growth of *L. leichmannii*, which resulted in higher OD<sub>530</sub> values (Fig. 2). These results indicate that vitamin B<sub>12</sub> uptake has been blocked in the resistant mutants due to defective *btuB* (interrupted by the IS elements). EC<sup>Cr</sup> and SS<sup>Cr</sup> complemented with *pbtuB* showed vitamin B<sub>12</sub> uptake similar to those of the susceptible mutants, thereby highlighting the marked physiological effect of the mutation (Fig. 2).

**Estimation of the mutation rate using the fluctuation assay.** A large variation in the number of colicin-resistant colonies in a parallel series of small cultures compared with that in a series of samples taken from the bulk culture is in agreement with the fact that mutations have occurred spontaneously (see Tables S1 and S2 in the supplemental material). The rates at which EC<sup>Cr</sup> became resistant to colicins E3 and E8 and colicins E3 and E2 were estimated to be  $2.51 \times 10^{-8}$  and  $5.52 \times 10^{-8}$  per generation, respectively. Further, we determined the percentage of those mutations that involved IS insertions. In the case of EC<sup>Cr</sup> selected after exposure to E3 and E8 colicins produced by the strain IDH01157, 10.3% ( $n = 300$ ) of the mutants exhibited a disruption of *btuB* by IS elements, whereas this figure was higher (18.7%,  $n = 300$ ) in the case of the EC<sup>Cr</sup> mutants selected on plates containing E2 and E3 colicins produced by the strain 500867.

## DISCUSSION

In the past 5 years (2008 to 2012), the frequency of the occurrence of *S. sonnei* strains as a sole pathogen (58%,  $n = 83$ ) among diarrheal patients admitted to the Infectious Diseases Hospital, Kolkata, has increased considerably. This provided us with an impetus to screen *S. sonnei* strains for colicin production (see Table S3 in the supplemental material). As anticipated, colicinogeny was confirmed in 92% of the strains. The majority of the *S. sonnei* strains isolated produced the E type of colicins (93%, 39 of 42 strains). To our knowledge, this is the first report on the occurrence of such a high frequency of E colicin-producing *S. sonnei* strains. The data obtained upon integration of phenotypic and genotypic methods revealed that most of them were E3 colicin producers. Among the few multicolicin producers, colicin E3 was found to co-occur either with colicin E2 or E8. Weak colicin producers seem to be very common (28). There is a possibility that the colicin types expressed by each strain are underestimated, as our screening is chiefly based on PCR and colicin assays. The multiple colicin production property confers a selective survival advantage not only against sensitive cells in a population but also against those harboring one of the colicins common to the multiproducer (3). The dynamics between colicin producers opens an interesting area of research.

There are very few reports on the role of *S. sonnei* colicins in governing the existence of shigellae and DEC. Considering this point, we tested the antagonistic effect of colicin-producing *S. sonnei* against a wide panel of DEC groups. The antagonistic spectrum suggests that most of the *S. sonnei* strains produced colicins that were biologically active against *E. coli* strains belonging to different DEC groups. Furthermore, *S. sonnei* strains exhibited conspecific as well as interspecific antagonism. This was evident from the hospital-based surveillance study. *S. flexneri* 2a and *S. dysenteriae* type 1 were specifically chosen for testing of their susceptibility to colicin because of an upsurge of multidrug resistance in these two strains (29), which demands a change in the antibiotic-based treatment regimen. Further, *S. flexneri*, particularly serotype 2a, is the serotype most endemic in Kolkata (30). However, during the past 2 decades, shigellosis caused by *S. sonnei* has become an emerging trend in India and other Asian countries (31–33). There are many reports on *S. sonnei* replacing *S. flexneri* as the predominant agent of bacterial dysentery in Vietnam, Thailand, Malaysia, China, and several other countries undergoing economic expansion (34). Epidemiologically, this is an important trend, as each *S. sonnei* strain generates its colicin(s), leading to competition that eventually reduces the frequency of the other species of bacteria belonging to the family *Enterobacteriaceae* (35).

The strategy employed by *S. sonnei* strains to direct the “microbe-kill-microbe” world in which they live provides an impetus to employ *S. sonnei* colicins for controlling the DEC, but paradoxically, just as colicin production is ubiquitous, so is resistance to colicin killing. However, as these toxins have a narrow spectrum of activity, the selection for resistance does not take place in several strains simultaneously. Further, colicins occur in constantly evolving combinations in nature, thereby enabling the colicin producers to keep pace with the emergent resistant ones. In addition to the emergence of resistance, the impact of the toxin on the commensal microbiota also deserves equal attention, before therapeutic applications commence. Several studies have shown that

colicins leave the structure of the commensal microbiome largely undisturbed (18, 19). In our case, we found that the majority of commensal *E. coli* strains (85%,  $n = 20$ ) were colicin resistant. Given the recent upsurge in publications highlighting the beneficial impact of commensal organisms, it is important to formulate therapeutic strategies for preserving commensal microbiota, and colicins can be exploited to that end. The limited toxicity of colicins to commensal organisms compared to that to enteric pathogens is very interesting and may be accounted for by the differences in the genetic makeups of the two groups of microorganisms. Several groups are actively pursuing research to address this long-standing question.

It has already been proven that a mutation in the *btuB* gene is responsible for instigating resistance to E colicins. However, there are no reports on the genetic mapping of the definitive locus within the *btuB* gene involved in the generation of resistance. We have focused primarily on the mutations that map *btuB* instead of *tolQRAB*, since previous studies have shown that the majority of the mutants resistant to E colicins (E2 to E8) possess mutations in the *btuB* gene (36). Our studies prove that the IS element-mediated disruption of the *btuB* gene is one of the mechanisms imparting colicin resistance. The classical Luria-Delbrück fluctuation test gave us an insight into the spontaneous nature of mutagenesis. Mutations occurring at an early stage in the small cultures gave rise to “jackpots” of resistant colonies on selective plates, thereby accounting for the high statistical variance (37). Had the mutations been induced by colicin, all mutants would appear after plating and yield a low variance in the number of resistant mutants.

Insertion sequences are mobile elements, which have a marked influence on the phenotype of an organism. These elements carry information required only for their mobilization, and they range in size from 0.7 to 2.5 kb (38–40). After exposing the EC<sup>Cs</sup> and SS<sup>Cs</sup> strains to a crude preparation of *S. sonnei* colicin, we found IS1 and IS2 disrupting the *btuB* gene in *E. coli* and IS1 and IS911 in *S. sonnei*. IS1 is one of the smallest “autonomous” bacterial insertion sequences isolated so far (770 bp) (41). Both IS2 (1,300 to 1,350 bp) and IS911 (1,250 bp) are members of the IS3 family, and their insertions have been reported in both the regulatory and coding regions (38). Insertional events have been reported to trigger resistance to bacteriophages (10) and to antibiotics (42, 43). However, to our knowledge, this is the first report on insertion sequences acting as one of the determinants in imparting colicin resistance by disrupting the *btuB* gene. We found that complementation of EC<sup>Cr</sup> and SS<sup>Cr</sup> with intact *btuB* resulted in the restoration of a colicin-susceptible phenotype and the complemented strains exhibited a degree of vitamin B<sub>12</sub> uptake similar to those of the wild-type progenitors. Further studies are warranted to interpret the changes that the BtuB protein undergoes upon disruption of its encoding gene.

Taking into account the fact that colicins seem to play a vital role in regulating the DEC, we hypothesize that the domination by *S. sonnei* occurs by competitively excluding the colicin-susceptible pathogens. The propensity for resistance development against colicin may be limited if a cocktail of colicins targeting different receptors is used. In addition, understanding the mechanisms behind the emergence of resistance will enable researchers to combat this problem more efficiently.

## ACKNOWLEDGMENTS

This work was supported by the Council of Scientific and Industrial Research (CSIR), New Delhi, India. F.C. is a recipient of an SPM award for carrying out this study under fellowship grant SPM-07/482(0087)2010-EMR-I.

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