Organization of the *Escherichia coli* and *Salmonella typhimurium* chromosomes between flagellar regions IIIa and IIIb, including a large non-coding region

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Flagellar regions IIIa and IIIb of the *Escherichia coli* and *Salmonella typhimurium* chromosomes (at 40 min and 42–43 min, respectively) has been shown to be separated by DNA unrelated to flagellar function, with region IIIa being immediately followed by a gene, *amyA*, that encodes a cytoplasmic α-amylase. The chromosome between *amyA* and flagellar region IIIb has now been investigated. The high level of DNA similarity between the *E. coli* and *S. typhimurium* sequences that exists in flagellar region IIIa and in *amyA* continues initially, with three genes of unknown function; in *E. coli*, there may be a fourth gene. The remainder of the region, up to the start of flagellar region IIIb, lacks any obvious open reading frames, scores poorly on an algorithm for coding probability, has a high A+T content, and is totally dissimilar in the two species. We conclude that it is non-coding. In *E. coli* this region extends for 2.7 kb and in *S. typhimurium* for 0.8 kb. These values are unusually large for prokaryotes, where the non-coding regions between operons are generally quite short. The data, which are discussed in the context of a hypothesized disruption of a contiguous ancestral flagellar region, may give new insight into the organization and evolution of the bacterial chromosome.

**Introduction**

In contrast to eukaryotic genomes, bacterial genomes are compact, with typically only around 50–300 bp of non-coding sequence between adjacent operons. For example, in the flagellar regulons of *Salmonella typhimurium* and *Escherichia coli*, (subscripts E and S are used throughout this paper to distinguish *E. coli* and *S. typhimurium* genes) among the cases where the non-coding distance between adjacent operons has been established, the mean value is 151 bp, the highest value is 263 bp (between the *flIC* and *flID* operons of *E. coli*; Hanafusa et al., 1989), and the lowest value is 64 bp (between the *flgB* and *flgK* operons of *S. typhimurium*; Homma et al., 1990).

We showed recently (Kawagishi et al., 1992) that a set of 19 flagellar genes in *E. coli*, which originally had been called flagellar region III, in fact consists of two contiguous sets (region IIIa, containing 5 genes, and region IIIb, containing 14 genes) separated by about 6.6 kb; a similar situation exists in *S. typhimurium* except that the intervening region is smaller (4.4 kb). Thus the last operon in flagellar region IIIa and the first operon in flagellar region IIIb are separated by a large distance. What is the nature and function of this DNA? Chromosomal deletion established that it contains no genes necessary for motility or for normal growth rates on minimal medium, but of course this does not exclude the possibility that it contains non-essential genes. Indeed, this intervening region is so large that it would be surprising if it was entirely non-coding.

The supposition that it would contain at least some coding sequence was soon vindicated by the finding that the last flagellar operon in region IIIa (the *flID* operon) was immediately followed by a gene, *amyA*, which encodes a cytoplasmic α-amylase (Raha et al., 1992). We have now examined the entire intervening region, and conclude that although it does contain several genes, it also contains an unusually long stretch of non-coding sequence.

**Methods**

*Bacterial strains.* *E. coli* strains DH5α (Gibco BRL) and XL1-Blue and JM101 (Stratagene) were used for cloning, subcloning and M13 production.
Cloning and sequencing of E. coli DNA. pIK1001 is a pBR322-based plasmid with an insert that contains the fliDE operon of flagellar region IIIa, the entire intervening region, and flagellar region IIIb genes from fliE to fliG through an EcoRI site in fliG (Kawagishi et al., 1992). A 2.1 kb fragment of the insert between the MluI and XbaI sites was subcloned into pBluescript (International Biotechnologies Inc.) to give plasmid pMR7. Using the XbaI site and the vector HindIII site, the cloned fragment from pMR7 was recloned into the corresponding sites in plasmid Bluescript plasmid pMR8.

The approaches used to sequence amyA, and the DNA up to a MluI site 2.2 kb beyond amyA have been described (Raha et al., 1992), as have those for the DNA from an XbaI site 0.7 kb before fliE by the dideoxy chain termination method using commercial synthetic primers. Both the MluI and XbaI sites were sequenced through, using appropriate plasmids.

Cloning and sequencing of S. typhimurium DNA. pOYAl is a pUC119-based plasmid (Müller et al., 1992); it has a 6.7 kb EcoRI-EcoRI insert of S. typhimurium DNA that extends from within fliD in flagellar region IIIa through all of the intervening region and as far as fliE in flagellar region IIIb. The approaches used to sequence amyA, and fliE have been described (Raha et al., 1992; Müller et al., 1992). For sequencing the rest of the intervening region, four M13 derivatives of pOYAl were used. The first two contained a Clal-Hpal fragment (from within amyA) to near the end of fliE cloned into M13mp18 and M13mp19, while the second two contained a BamHI-EcoRI fragment (from within fliE to fliG) cloned into the same vectors.

Chemicals and enzymes. All chemicals and enzymes were obtained from standard commercial sources. Antibiotics were used at the following concentrations: ampicillin, 50 μg ml⁻¹; tetracycline, 10 μg ml⁻¹. Synthetic primers (18-mers) were synthesized using an Applied Biosystems 392 DNA/RNA Synthesizer.

Results

Overall organization of the intervening regions in E. coli and S. typhimurium

The fliD operon in flagellar region IIIa is followed by an amylase gene, amyA, oriented clockwise on the chromosome (Raha et al., 1992). We have now extended the sequence analysis all the way through the intervening region to the 3' end of fliE (see Methods for details). The results are shown schematically in Fig. 1(a); the corresponding DNA sequence is available in GenBank under accession number L13279. The fragment used for analysing amyA contained a second open reading frame oriented counterclockwise, with a short (77 bp) distance between the 3' ends of the respective coding regions. This second open reading frame has a deduced product molecular mass of 15 kDa, and will be referred to as orf15. Diverging from the 5' non-coding region of orf15 were two open reading frames with an ATGA overlap between them; both had strong consensus ribosome binding sequences. They correspond to products with deduced molecular masses of 48 and 9 kDa, respectively, and so will be referred to as orf48 and orf9. About 100 bp following the 3' end of orf9 was a fourth short open reading frame, orf8, with an extremely strong, well-placed consensus ribosome binding sequence (AGGAGG), and followed by a pronounced stem-loop sequence. Based on their opposite orientation, amyA, orf15 and orf48 must belong to distinct transcriptional units. With a 4 bp overlap of their coding regions, orf48 and orf9 presumably belong to the same transcriptional unit, while the distance between the 3' end of orf9 and the 5' end of orf8 suggests that the latter is a separate transcriptional unit. It is not obvious from inspection what the corresponding promoters are and, in the absence of knowledge regarding gene product function (see below), we did not attempt to determine the transcription start sites.

For the next 2.7 kb, until the first flagellar gene in region IIIb, fliE, there was no evidence for any further genes: open reading frames in both orientations were very short (mostly less than 300 bp) and had no obvious ribosome binding site.

The intervening region of S. typhimurium followed a similar pattern (Fig. 1(b); sequence available in GenBank under accession number L13280), with two exceptions: there was no evidence for an open reading frame corresponding to orf8, and the distance between the last open reading frame, orf9, and fliE was much shorter (0.8 kb) than in E. coli.

Comparison of the DNA sequence of the intervening region in the two species

When DNA sequences of the intervening region in E. coli and S. typhimurium were compared in a matrix plot (Fig. 2), the similarity seen previously in flagellar region IIIa and amyA was found to extend through orf15, orf48 and orf9. These open reading frames in the two species show 77, 81 and 86% identity, respectively, when aligned by the algorithm of Lipman & Pearson (1985).

Following orf9, no sequence similarity was discerned, either by inspection of Fig. 2 or by attempted alignment, until the start of flagellar region IIIb. Thus orf8 plus the 2.7 kb between orf8 and fliE is unrelated to the 0.8 kb between orf9 and fliE. If they were part of the same sequence originally, they must since have diverged beyond recognition, and also (at least in the case of S. typhimurium) undergone further rearrangement by deletion.

Evidence that orf15, orf48 and orf9 are authentic genes

Minicell analysis (Raha et al., 1992) has demonstrated that orf15 encodes a 15 kDa protein and so is an authentic gene. We conclude that orf48 and orf9 are
authentic genes also, based on the following considerations.

The strongest evidence is that the deduced amino acid sequences show even higher levels of identity than the corresponding nucleotide sequences (83, 92 and 100% for Orf15, Orf48 and Orf9, respectively; Fig. 3). This result is statistically extremely improbable for non-coding DNA, because of the much larger basis set of 20 amino acids vs. that of four nucleotides. Inspection of the genetic code reveals that a one-base codon change has a probability of 0.76 of causing an amino acid change, and so between closely related sequences (where changes of more than one base per codon can be neglected) an x% difference in nucleotide sequence (corresponding to a 3x% difference in codons) will statistically yield an amino acid difference of 2.3x%. In the more general case where multiple changes per codon cannot be neglected, computer simulation demonstrates that translation of two DNA sequences which differ from each other by random changes to a given extent (say, 20%) generates deduced amino acid sequences that are much more different (in this case about 40%).] As expected from the genetic code, the nucleotide alignments for orf15, orf48 and orf9 show that mismatches at the
third-base position are much more prevalent than mismatches at the other two positions, and are the major reason for the high amino acid similarity levels.

The conclusion from comparisons between *E. coli* and *S. typhimurium*, namely that these open reading frames correspond to real genes, is independently supported by evidence deriving from the individual sequences: (i) the open reading frames were scored as coding regions by the algorithm TTESTCODE (Fickett, 1982) (see below) at confidence levels of 96, 96, 88, 95, 99 and 96% for *orf15*, *orf48*, *orf9* (and also *orf8*), respectively; and (ii) they all have strong, well-placed consensus ribosome binding sequences. *orf9* also appears to be an authentic gene by these criteria, and has a TTESTCODE score of 96%.

The *Orf15*, *Orf48*, *Orf9* (and also *Orf8*) sequences were compared against the GenBank database (version 75.0, February 1993) using TFASTA, but no significant similarities were found. In the absence of any clues to the functions of these proteins, we have not attempted to further identify and characterize them, but note briefly below some distinctive characteristics about their deduced amino acid sequences.

*Orf15*. This sequence has a basic N-terminus followed by a hydrophobic region which is, however, too short to be membrane-spanning. The C-terminus has the sequence TPLP repeated three times in the *S. typhimurium* sequence and (as APLPTPLP) twice in the *E. coli* sequence. C-terminal repeats involving T/S and P (but not of the precise form seen in *Orf15*) have been noted in a variety of DNA binding proteins, including RNA polymerase II and histones (Suzuki, 1990).

*Orf48*. This sequence is quite basic, with a total of 39 K + R + H residues compared with 17 D + E residues (*E. coli* data). The N-terminus is basic and is followed by the first of several (seven or eight) hydrophobic regions that are predicted to be membrane-spanning by the algorithm of Engelman et al. (1986). These extend throughout the entire sequence, and suggest that *Orf48* is an integral membrane protein with no substantial aqueous domains.

*Orf9*. No special features were evident for this sequence, which appears to be that of a small soluble protein.

The apparently non-coding region immediately prior to *fliE*

As was described above, *E. coli* and *S. typhimurium* have 2·7 and 0·8 kb of DNA following a series of open reading frames and prior to the first gene of flagellar region IIIb, *fliE*. The absence of open reading frames of significant size (and also the complete lack of any sequence similarity between the two species) indicated that the DNA was non-coding.
To test this further, we used the algorithm TESTCODE devised by Fickett (1982). This is based on autocorrelation analysis of nucleotides at step 3, and scores the probability that DNA sequence is coding. No knowledge of frame is required, no attention is paid to stop codons, and the algorithm does not depend on signals such as ribosome binding consensus sequences. Typically (e.g. the open reading frames described above), authentic coding sequence scores as such with a confidence level of 90% or higher, whereas non-coding sequence scores very poorly. The algorithm can also be run using a scanning window: in this case, when an authentic reading frame is scanned the confidence level is typically in the 90th percentile most of the time with occasional downward fluctuations; in contrast, 5' and 3' non-coding sequences score as coding at around the 50-20th percentile confidence level, with occasional upward fluctuations.

When the *E. coli* DNA sequence spanning flagellar region IIIa, the intervening region, and flagellar region IIIb was examined in this way, the results were striking (Fig. 4a). All the way from the flagellar region IIIa genes through *orf8* (except for the non-coding regions between adjacent operons) coding probabilities were high; the same was true in flagellar region IIIb. Between *orf8* and *fliE*, however, the coding probabilities were for the most part extremely low; there were a few spikes of higher probability, which might be statistical fluctuations or might represent local regions where coding information has not been entirely lost. A similar situation applied to *S. typhimurium* (Fig. 4b), but in this case the high probability region corresponding to *orf9* was lacking and the predicted non-coding region was much shorter, as the similarity matrix in Fig. 2 had already revealed.

Further evidence for absence of a coding function for this DNA came from base composition. Whereas coding sequence of DNA in *E. coli* and *S. typhimurium* commonly has an A+T content of around 48%, control and other non-coding regions generally have a higher A+T content, often as high as 65% (Haughn et al., 1986). This was found to be true of the 2.7 kb region between *orf8* and *fliE*, and the 0.8 kb region between *orf9* and *fliE*, where the mean A+T content was 59% in both cases (data not shown). The later part of the 2.7 kb
region in E. coli was especially AT-rich, with a mean content of 66% over more than 14 kb.

In a search of the GenBank database using FASTA, we found no significant similarities to these non-coding sequences; the highest scoring examples (optimized scores in the range 100–150) were AT-rich regions from a variety of sequences.

The DNA in this region lacks any distinguishing characteristics that might provide a clue to its function. It contains some examples of inverted repeats scattered throughout the sequence, but probably not more than would be expected by chance; it does not contain any major direct repeats. No examples of the repetitive extragenic palindromic (REP) sequences that are scattered around the chromosomes of E. coli and S. typhimurium were found within these apparently non-coding regions; this result agrees with the physical mapping of REP sequences throughout the entire E. coli genome (Dimri et al., 1992), where the only one in the vicinity of flagellar regions IIIa and IIIb is REP40 at the 3' end of fliC (Hanafusa et al., 1989).

Discussion

In this study, we have attempted to understand the organization and significance of the several kilobases of DNA that separates two major clusters of flagellar genes in both E. coli and S. typhimurium (Fig. 1 a, b).

The fact that flagellar regions IIIa and IIIb are within only a few kilobases (i.e. about 0.1% of the chromosome) of each other suggests to us that originally they were contiguous and then were subjected to rearrangement by insertion of DNA, either from elsewhere on the chromosome or from another source such as a plasmid. If this is true, the rearrangement must be an ancient one that occurred before the speciation of E. coli and S. typhimurium around 140 million years ago (Ochman & Wilson, 1987). What is the nature of the inserted DNA, and what has happened to it in the time since it disrupted the ancestral flagellar region?

The intervening region was known to contain at least one gene, which codes for a cytoplasmic α-amylase, AmyA (Raha et al., 1992). The analysis of the entire region in the present paper has revealed several other genes. Database comparisons provide no basis for suggesting that they play a role in sugar metabolism and so it appears that amyA exists as an isolated gene remote from others that may have related roles, such as the glg (glycogen) cluster located at 76 min on the E. coli map (Bachmann, 1990).

Although the functions of orf15, orf48 and orf9 are unknown, for several reasons we are confident that they are authentic genes. The most compelling argument is the very high level of amino acid identity for the deduced products in the two species. orf8, present only in E. coli, is probably an authentic gene also, although here we do not have the amino acid comparison to rely on.

The transition from the last flagellar region IIIa operon to amyA and the other open reading frames described above is abrupt, with a typically short non-coding region. The same is true between amyA and orf15, between orf15 and orf48, and (in E. coli) between orf9 and orf8. From that point on, all the way to the boundary of flagellar region IIIb, the DNA sequence appears to be non-coding. There are four lines of evidence for a predominantly or exclusively non-coding function, which taken together are compelling: (i) the DNA contains no open reading frames of significant length; (ii) the DNA scores very poorly as coding sequence; (iii) the A+T content is high, a characteristic of non-coding sequence; and (iv) there is no detectable similarity between the E. coli and S. typhimurium sequences. The existence of substantial amounts of non-coding DNA at the rightmost end of the intervening region is in striking contrast with the extremely tight apposition at the leftmost end, where apparently all redundant DNA between the region IIIa flagellar genes and amyA has been squeezed out. Why the two ends of the intervening region should be so different is unclear.

We have examined the literature for other known examples of substantial regions of non-coding DNA on the E. coli or S. typhimurium chromosomes. Haughn et al. (1986) reported a 500 bp gap between two divergent genes, leuP and an open reading frame of unknown function. More recently, as part of the ongoing E. coli genome project, Daniels et al. (1992) have described the sequence of 91 kb of DNA between 84.5 and 86.5 min on the chromosome; within that sequence they have found examples of large regions of apparently non-coding DNA, and have coined the phrase ‘grey holes’ for such regions. Interestingly, in another recent report of an extensive (111 kb) contiguous sequence of the E. coli chromosome, from 0 to 24 min, no such grey holes were found (Yura et al., 1992). The largest grey hole described by Daniels et al. was 1–2 kb. The one between flagellar regions IIIa and IIIb is, at 2.7 kb, more than twice as large and is to our knowledge the largest that has been found thus far on the E. coli chromosome.

It is not known whether grey holes represent sequence that performs a useful function such as, for example, stabilizing higher-order chromosome structure. At least in the case of the grey hole between flagellar regions IIIa and IIIb, it is hard to imagine it having any such function, given its disparate sizes in E. coli and S. typhimurium.

If these extensive stretches of DNA are non-functional, how might they arise? Genetic rearrangements such as random insertions or deletions will, in general, cause
flagellar assembly process, while those in region IIIb are different, with those in region IIIa being involved in the switch and the basal-body MS ring. Perhaps this differentiation is in some way related to the events that have caused the physical separation on the chromosome.

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References


