

# Expression of the *Escherichia coli dam* gene

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## Summary

The *Escherichia coli dam* gene and upstream sequences were cloned from the Kohara phage 4D4. Five promoters were found to contribute to *dam* gene transcription. P1 and P2 (the major promoter) were situated approximately 3.5 kb upstream of the structural gene, P3 was within the *aroB* gene, P4 was within the *urf74.3* gene, and P5 was in the *urf74.3-dam* intergenic region. The nucleotide sequence of 2280 bp of DNA containing P1 and P2 was determined and shown to have the potential to encode a protein of approximately 16 kDa between P1, P2, and the *aroB* gene. This 16 kDa open reading frame has been identified as *aroK*, the gene for shikimic acid kinase I. Thus the *dam* gene is part of an operon containing *aroK*, *aroB*, *urf74.3*, and *dam*. The transcriptional start points of the promoters were determined. A comparison of their nucleotide sequences suggested that P1–P4 were all recognized by the  $\sigma^{70}$  subunit of the RNA polymerase.

## Introduction

The *Escherichia coli* Dam (DNA adenine methyltransferase) protein recognizes the sequence GATC in double-stranded DNA and methylates the adenine residue at the N6 position (Lacks and Greenberg, 1977; Hattman *et al.*, 1978). The Dam methyltransferase functions as a single polypeptide of 32 kDa (Herman and Modrich, 1982) that is encoded by the *dam* gene located at 74 min on the genetic map (Marinus, 1973; Bachmann, 1990). Methylation of GATC sequences is a post-replicative process and occurs

<1.5–10 min after passage of the replication fork, depending upon surrounding sequences (Campbell and Kleckner, 1990). The time from replication to methylation is a period when the DNA is hemimethylated, i.e. methylated on one strand only, in contrast to the remainder of the DNA which is fully methylated. The transient hemimethylation of newly replicated DNA activates or suppresses certain processes in the cell. Since hemimethylation always follows immediately after passage of the replication fork, these processes are linked to the cell cycle (Messer and Noyer-Wiedner, 1988; Barras and Marinus, 1989).

Mutations in the *dam* gene lead to ultraviolet (u.v.) radiation sensitivity, increased mutability, and to inviability in combination with mutations in genes such as *recA*, *lexA*, and *polA* (Marinus and Morris, 1975; McGraw and Marinus, 1980). This strongly suggests the involvement of *dam* methylation in DNA repair processes. The function for methylation in the repair process seems to be a strand discriminatory role (Herman and Modrich, 1981; Marinus *et al.*, 1984; Modrich, 1987; Meselson, 1988).

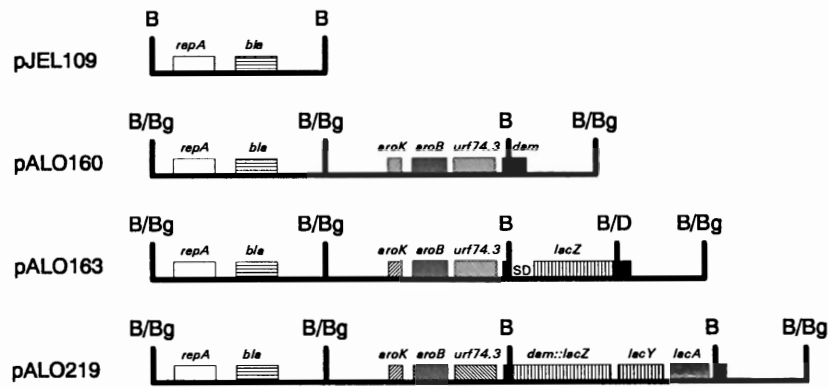
Dam methylation affects processes such as regulation of gene expression (Roberts *et al.*, 1985; Braun and Wright, 1986; Schauzu *et al.*, 1987; Peterson *et al.*, 1985; Marinus, 1985), the packaging of bacteriophage P1 DNA into virions (Yarmolinsky and Sternberg, 1987), and functions associated with bacterial retroposons (Hsu *et al.*, 1990).

Dam methylation also influences the initiation of chromosome replication from *oriC*. The transient hemimethylation of *oriC* following replication was shown to block new initiations (Russell and Zinder, 1987). The hemimethylated stage is probably responsible for limiting initiation of each chromosomal origin of replication to once per cell cycle (Boye and Løbner-Olesen, 1990). This eclipse period for initiation may be accomplished by sequestering the hemimethylated origin into the cell membrane, thus anchoring it to allow segregation of chromosomes to daughter cells (Ogden *et al.*, 1988).

The rate of methylation of newly replicated GATC sequences obviously has a profound influence on all of the processes described above, which suggests that the level of Dam methylase in the cell must be carefully regulated. This, however, has never been tested experimentally, and virtually nothing is known about regulation of the *dam* gene.

Upstream of *dam* is the *aroB* gene and an unidentified open reading frame (*urf74.3*) that is located between the

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**Fig. 1.** Structure of the plasmids pJEL109, pALO160, pALO163, and pALO219. The plasmids were constructed as described in the *Experimental procedures*. Plasmid pJEL109 is the cloning vector. Plasmid pALO160 is pJEL109 containing the 8 kb *BglII* fragment carrying the *dam* gene from phage lambda 4D4 inserted into the unique *BamHI* site. Plasmid pALO163 was derived from pALO160 by inserting the *lacZ* gene from plasmid pTL25 into the unique *BamHI* site of plasmid pALO160, thus generating a *dam-lacZ* transcriptional fusion plasmid. Plasmid pALO219 is a *dam-lacZ* translational fusion plasmid derived from pALO160 by fusing the *lacZ* gene in frame to the *dam* gene using the unique *BamHI* site carried by plasmid pALO160. Restriction enzyme cleavage sites: B, *BamHI*; Bg, *BglII*; D, *DraI*. SD is the Shine-Dalgarno sequence of the *lacZ* gene.

*aroB* and *dam* genes (Jonczyk *et al.*, 1989). In this region, three promoter elements have been proposed on the basis of studies using high copy-number plasmids: one promoter immediately upstream of *aroB* (Millar and Coggins, 1986); one promoter within *aroB* (Jonczyk *et al.*, 1989); and one weak promoter immediately upstream of the *dam* gene (Arraj *et al.*, 1990; Wu *et al.*, 1992). Here we present evidence that *aroB*, *urf73.4*, and *dam* all belong to the same transcriptional unit. By using single copy-number plasmids we have defined five promoter elements in the operon, the strongest of which is located 3.2 kb upstream of the *dam* gene. As a first step towards elucidating the mechanisms of *dam* gene regulation we have located and sequenced the chromosomal region resulting in transcription of the *dam* gene.

## Results and Discussion

### *Cloning of the dam region and construction of dam-lacZ fusion plasmids*

Bacteriophage lambda 4D4 (Kohara *et al.*, 1987) was used as the source of DNA for cloning of the *dam* and contiguous genes to produce the low copy-number plasmid, pALO160 (Fig. 1). This plasmid complemented mutations in the chromosomal *dam* and *aroB* genes. Transcriptional (pALO163) and translational (pALO219) fusions of *lacZ* to the *dam* gene were used to study *dam* gene expression (Fig. 1). We have used a low copy-number plasmid in our experiments because preliminary work indicated that clones containing the region upstream of *dam* on high copy-number plasmids contained deletions and rearrangements. At present we know neither the exact position of the unstable sequence nor the reasons for its instability in high copy-number plasmids.

### *Mapping of the dam promoters by deletion analysis*

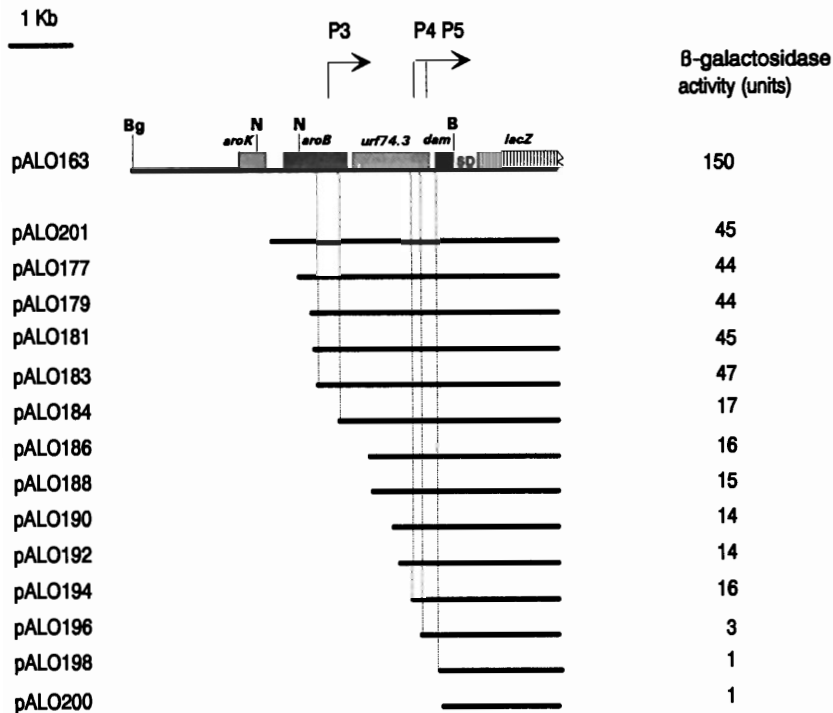
Plasmid pALO163 was used as the basis for constructing a series of plasmids containing deletions stretching from

the left-most *BglII* site at position 1 in the sequence to the *BamHI* site in the *dam* gene (Fig. 2). The extent of the deletions was established by nucleotide sequencing. The corresponding levels of  $\beta$ -galactosidase (Fig. 2) showed a large difference between plasmids pALO183 and pALO184, as well as between plasmids pALO194 and pALO196. These four plasmids thus delimit two potential promoter regions termed P3 (situated between nucleotides 3013 and 3372) and P4 (between 4611 and 4751; for sequence numbering, see the *Experimental procedures*). Yet another promoter, termed P5, positioned closer to the *dam* gene has already been characterized (Arraj *et al.*, 1990; Wu *et al.*, 1992).

The amount of  $\beta$ -galactosidase produced with plasmids pALO177 and pALO201 (Fig. 2) was essentially the same, suggesting that the *aroB* gene is not immediately preceded by a promoter region, which is in contrast to an earlier report (Millar and Coggins, 1986). In support of our observation, it was found that plasmid pALO201, which contains the whole coding sequence for *aroB*, was unable to complement the *aroB* mutation in strain AB2847, whereas plasmid pALO163 did.

The activity of  $\beta$ -galactosidase produced with the parental plasmid, pALO163, was approximately three times higher than that produced with plasmid pALO201 (Fig. 2). Therefore the major promoter(s) for the *dam* gene is (are) situated within the *BglII-NruI* fragment that was deleted from plasmid pALO163 to give rise to plasmid pALO201.

In order to map this major promoter, another set of deletion derivatives of plasmid pALO163 was made. These deletions all started at the *BamHI* site in the *dam* gene and extended upstream for varying distances (Fig. 3). The  $\beta$ -galactosidase activities in cells containing these plasmids suggested the presence of two additional promoter regions delimited by the endpoints of plasmids pALO205 and pALO214 (P1) and plasmids pALO214 and pALO213 (P2). This places P1 between positions 1504 and 1739 and P2 between positions 1739 and 1939. The

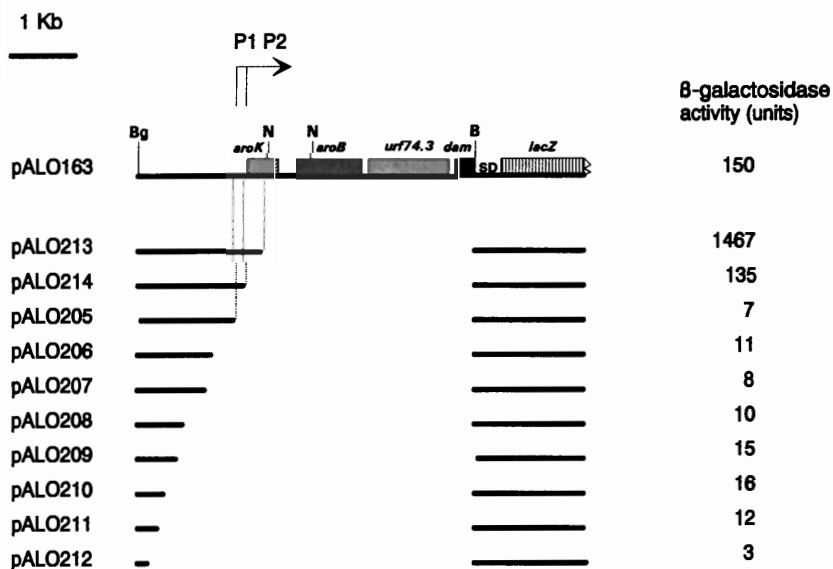


**Fig. 2.** Identification of *dam* promoters P3, P4, and P5. Deletion analysis of the *dam* promoter region. A map of the promoter region on the *dam-lacZ* transcriptional fusion plasmid, pALO163, is shown at the top. All plasmids except pALO201 were constructed by nuclease Bal31 treatment of *Nru*I-digested pALO163, followed by recloning into plasmid pJEL109. Plasmid pALO201 was constructed by the use of restriction enzymes only. For details of all constructions see the *Experimental procedures*. The level of  $\beta$ -galactosidase activity conferred by the plasmids in strain LJ24 is shown on the right. P3, P4, and P5 represent potential promoter regions. The position of P5 was determined by Arraj *et al.* (1990) and Wu *et al.* (1992). Restriction enzyme cleavage sites: B, *Bam*HI; Bg, *Bg*II; N, *Nru*I.

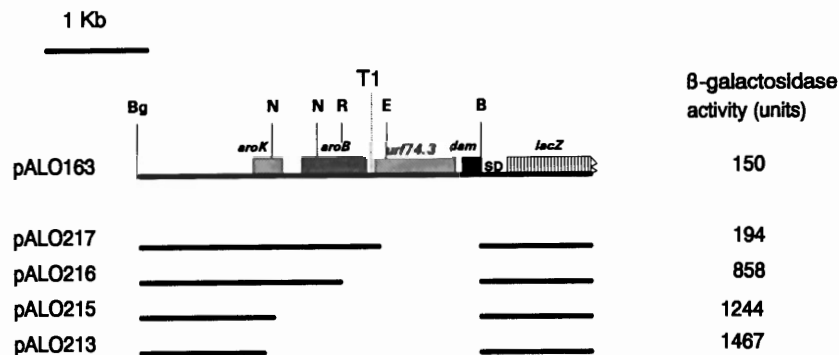
amounts of  $\beta$ -galactosidase produced by plasmids pALO213, pALO214, and pALO205 suggest that P2 is the stronger promoter and responsible for the major part of *dam* gene transcription.

Our results on the location of promoter elements confirm the existence of a previously described promoter but are at variance with regard to another. Jonczyk *et al.* (1989) identified a promoter region within the *aroB* structural gene on the basis of increased reporter gene activity. The location of this promoter activity (based on the

position of restriction endonuclease sites) is almost certainly identical to our promoter P3. On the other hand, these workers did not find evidence for the existence of promoters P4 and P5. Millar and Coggins (1986) located a promoter immediately upstream of the *aroB* coding sequence. In the present work, however, we found no evidence of a promoter in this region. Our data indicate that *aroB* transcription starts from promoters P1 and P2, since low copy-number plasmids without promoters P1 and P2 fail to complement an *aroB* mutant. Furthermore,



**Fig. 3.** Identification of *dam* promoters P1 and P2. Deletion analysis of the *dam* promoter region. A map of the promoter region on the *dam-lacZ* transcriptional fusion plasmid pALO163 is shown at the top. All plasmids except pALO215 were constructed by nuclease Bal31 treatment of *Nru*I-digested plasmid pALO163, followed by recloning into plasmid pJEL109. For details of all constructions see the *Experimental procedures*. The levels of  $\beta$ -galactosidase conferred by the plasmids in strain LJ24 are shown on the right. P1 and P2 represent potential promoter regions. Restriction enzyme cleavage sites: B, *Bam*HI; Bg, *Bg*II; N, *Nru*I.



**Fig. 4.** Transcription termination between the *aroB* and *urf74.3* genes. A map of the *dam* promoter region transcriptionally fused to *lacZ* and carried by plasmid pALO163 is shown at the top. Deletion derivatives of plasmid pALO163 were constructed as described in the *Experimental procedures*. The level of  $\beta$ -galactosidase activity conferred by the plasmids in strain LJ24 is indicated on the right. T1 represents the *aroB* terminator proposed by Millar and Coggins (1986). Restriction enzyme recognition sites: B, *Bam*HI; Bg, *Bgl*II; N, *Nru*I; R, *Rsr*II; E, *Eco*RI.

insertion of a drug-resistance element 400bp upstream of *aroB* (at position 2059) results in aromatic amino acid auxotrophy because of a polar effect of the mutation on *aroB* expression (Løbner-Olesen and Marinus, 1992).

#### Mapping of transcriptional terminators

The  $\beta$ -galactosidase activity produced from plasmid pALO163 is only approximately 10% of that produced by plasmids pALO213 and pALO215 (Fig. 4). This means that the majority of transcripts arising from promoters P1 and P2 are terminated before reaching the *dam* gene. Approximately 40% of the original transcripts from these two promoters are terminated between nucleotides 2058 and 3083 (the *Nru*I site in *aroB* and the *Rsr*II site; compare the activities of plasmids pALO215 and pALO216 in Fig. 4). We have not been able to detect, by computer-aided analysis, any typical Rho-independent terminator structure in this region, and the mechanism of transcription termination remains obscure.

Of the remaining transcripts, approximately 80% are terminated between the *Rsr*II and *Eco*RI sites at nucleotides 3083 and 3635, respectively (compare activities of plasmids pALO216 and plasmid pALO217 in Fig. 4). This sequence includes the intergenic region between the *aroB* and the *urf74.3* genes, in which a stable stem-loop structure (designated T1 in Fig. 4) in the mRNA can be deduced from the nucleotide sequence (Millar and Coggins, 1986).

Based upon the deletion analyses in Figs 2, 3 and 4, the *dam* gene is transcribed from at least five promoters, and the majority of transcripts arising from the two distal promoters (P1, P2) is terminated in at least two different regions.

#### Nucleotide sequence of the region containing the major *dam* promoter

The two promoter regions P1 and P2 defined by deletion analysis were mapped to a region upstream of the *aroB* gene for which no sequence information was available. We

therefore determined the nucleotide sequence between the *Bgl*II and *Bal*I sites, and the total length of nucleotide sequence obtained was 2280bp (Fig. 5).

This sequence was merged with that of the *aroB* gene (Millar and Coggins, 1986) and analysed on both strands for open reading frames. It was found that nucleotides from position 1791 to 2222 had the potential to encode a protein of approximately 16 kDa (Fig. 5). This 16 kDa protein is expressed in the cell since a translational fusion to *lacZ* produces functional  $\beta$ -galactosidase (data not shown). The 16 kDa protein was identified as shikimic acid kinase I, and the gene symbol *aroK* has been assigned to this hitherto unknown gene (Løbner-Olesen and Marinus, 1992).

#### Promoter mapping by primer extension

In order to characterize the promoter regions in more detail, we mapped the transcriptional start points of the four previously unidentified promoters P1–P4 by primer extension analyses (Fig. 6). The lengths of the reverse transcripts indicated transcriptional starting points at nucleotides 1558 and 1771 (P1 and P2). The transcriptional starting point of P3 was mapped to nucleotide 3336, which is within the coding region of the *aroB* gene. The P4 starting point was located at nucleotide 4679 internal to the *urf74.3* gene. The previously characterized P5 transcript starts at nucleotide 4949 in the intergenic region between *urf74.3* and *dam* (Wu *et al.*, 1992).

The promoter sequences are shown in Fig. 7. With the exception of P5, all the promoters resemble classical RNA polymerase  $\sigma^{70}$  sequences, having –10 and –35 regions only slightly deviating from the consensus sequences TATAAT and TTGACA, respectively, and with a spacing of 16–18bp. Surprisingly, promoters P2, P3, and P4 give rise to transcripts beginning with a U. This is in contrast to the majority of *E. coli* promoters, in which the RNA polymerase initiates with A or G. Based upon the activities of *dam*–*lacZ* transcriptional fusions of plasmids containing one or more of the five promoters (Figs 2 and 3), we estimate the order of promoter strengths as being P2>P1>P3>P4>P5.

BglII  
 1 AGATCTATGC CGGATTAGCG AACTTAGCCA GTGGCGCTAT CAGGGGATGG  
 51 TAGGGCGAGG CGAGCGCATC ATCGGTGTAA TAAAGACGG GCAAATAGAA  
 101 TGGCGACGGG TGCAGCAAAA CGATGTGCTG GAAAACGGCT GGCAATTTT

pALO212  
 151 ACACGTCGAC CCAAGCCTAC CTTAACGCTG GGTACCGGGA CAAACTCGCA  
 201 ACCGCCACAA TGTTGTGGCC AACGGCAAGG AGATACAATG GAAGCAATGG  
 251 TAGTCCCGCA TACTGTGTAT GCTGATACGG GGCGTACAGG CGGCAAAAGCC

pALO211  
 301 GCAAAAAGTG ACGCTGATGG TGGATGACGT TCCGGTAGCT CAGGTGTTGC

pALO210  
 351 AGGCGCTGGC TGAACAGGAG AAGTTGAACC TGGTCGTGTC GGCAGACGTC  
 401 AGCGGTACGG TGTCGTTACA TCTAACAGAT GTTCCCTGGA AGCAGGCACT  
 451 ACAAACGTGA GTGAAAAGCG CCGGACTGAT AACCGGCGAG GAAGGCAACA  
 501 TTCTCTCAGT GCATTCATT GCCTGGCAGA ATAACAATAT CGCCGCCAGG

pALO209  
 551 AGGCGGAGCA GGGCGGGGCG CAGGCAATC TGCCGTGGA AAATCGCAGT  
 601 ATAACCTGCG AATACGCCGA CGCGGGAGAA CTGGCGAAG CGGGGGAGAA  
 651 GCTACTGAGT GCCAAAGGGA GTATGACCGT CGATAAACGC ACCAATCGCC

pALO208  
 701 TTTTGTACG AGATAACAAA ACGGCGTTAA GCGCGCTTGA ACAGTGGGTA  
 751 GCGCAAAATGG ATCTGCCGGT CGGGCAGGTT GAGCTGTGCG GCATATGTT  
 801 CACCAATTAAT GAAAAGAGTT TCGGTGAGTT AAGCGGTGAA ATGGACGCTG  
 851 GCCGATGCGC AACACGCTGG TGGCGTTGGG CAAGTCACCA CGGTTGGTAG  
 901 CGACCTCTCC GTAGCGGACG CGAACACGCA TGTCCGTTTT AACATTTGGG  
 951 GCGTCAACGG ATCGTTGCTG GATCTTGAAG TTTCCGCGCT GAAGCAAAAA  
 1001 CAGCAGCTGG ATATTATCGC CAGTCCGCGT CTGCTGGGCT CACATCTTCA

pALO207  
 1051 GCCTGCCAGC ATTAACAGG GGAGCGAAAT TCCATATCAG GTTCCAGCGG  
 1101 GGAAAGTGGC GCACGTCGGT GGAATTTAAA GAGCCCGTCC TGGGATGGAG  
 1151 GTCACGCCCA CGGTGTACCA AAAAGGTCCG ATCCGCGTTA AATTACACAT  
 1201 CAGCCTGAAC GTTCCGGGGC AGGTGCTACA CGAGCCCGAT GCGCAAGTGC  
 1251 TGGCGATTGA TAAGCAGGAG ATCGAAACGC AGTTCGAGT CAAAAGCGGA  
 1301 GAAACGTTGG CGTGGGGCG CATTTTTACC CGTAAATAA AATCGGGTGA  
 1351 GGATAGCGTA CCGTGTCTTG GCGACATTCC TGGTTCGGG CAATTATTTC  
 1401 GTCATGACGG AAAAGAGAT GAACGACGCG AGTTAGTGTG GTTATTCACG  
 1451 CCACGACTGG TTTCCAGTGA GTAACACGCG GTAAAAGCGG TAATGTTTTA

pALO205  
 1501 CGCTGAACGT GTTTCATCTA TTTGACGCGC GCAGGTATTT AGCATACAAG

→ P1 mRNA  
 1551 GAGTACCGAT TTAGAGTTG GTGCTCTTCG CTGCCTGCGT TCCATGATGA  
 1601 TGATTTATCA TTCAGGCGGC ATTTTGTCTG CTTTTTACG CTAATCTTAC  
 1651 CCGGTGATTT ATGCCACAGG CCGTGGTAGC AAGCAGCGC GCTTGCACGG

pALO214  
 1701 ACCAGATATG CAGAGGGATG GGTGATTTAT TCAGTTGCCA AACCCGCTGG

→ P2 mRNA  
 1751 AGTATTGAGA TAATTTTCAG TCTGACTCTC GCAATATCTT

1791 ATG AGG TTT CAG TTC ATG TCC TGC CGG GCC TCT CTG AGC GAA  
 MET Arg Phe Gln Phe MET Ser Cys Arg Ala Ser Leu Ser Glu

1833 GCG GGT TTA TCA TTA ACG AAT AGT CTT AGT AGT ACC GAA AAA  
 Ala Gly Leu Ser Leu Thr Asn Ser Leu Ser Ser Thr Glu Lys

1875 ATG GCA GAG AAA CGC AAT ATC TTT CTG GTT GGG CCT ATG GGT  
 MET Ala Glu Lys Arg Asn Ile Phe Leu Val Gly Pro MET Gly

pALO213  
 1917 GCC GGA AAA AGC ACT ATT GGG CGC CAG TTA GCT CAA CAA CTC  
 Ala Gly Lys Ser Thr Ile Gly Arg Gln Leu Ala Gln Gln Leu

1959 AAT ATG GAA TTT TAC GAT TCC GAT CAA GAG ATT GAG AAA CGA  
 Asn MET Glu Phe Tyr Asp Ser Asp Glu Ile Glu Lys Arg

2001 ACC GGA GCT GAT GTG GGC TGG GTT TTC GAT TTA GAA GGC GAA  
 Thr Gly Ala Asp Val Gly Trp Val Phe Asp Leu Glu Gly Glu

2043 GAA GGC TTC CGC GAT CGC GAA GAA AAG GTC ATC AAT GAG TTG  
 Glu Gly Phe Arg Glu Thr Arg Glu Lys Val Ile Asn Glu Lys Leu

2085 ACC GAG AAA CAG GGT ATT GTG CTG GCT ACT GGC GGC GGC TCT  
 Thr Glu Lys Gln Gly Ile Val Leu Ala Thr Gly Gly Gly Ser

2127 GTG AAA TCC CGT GAA ACG CGT AAC CGT CTT TCC GCT CGT GCG  
 Val Lys Ser Arg Glu Thr Arg Asn Arg Leu Ser Ala Arg Ala

2169 TTG TCG TTT ATC TTG AAA CGA CCA TCG AAA AGC AAC TTG CAC  
 Leu Ser Phe Ile Leu Lys Arg Pro Ser Lys Ser Asn Leu His

2211 GCA CGC AGC GGA TAA  
 Ala Arg Ser Gly

2226 AAAACGCCCG TTGCTGCACG TTGAAACACC GCCGCGTGAA GTTCTGGAAG  
 2276 CGTTG

**Fig. 5.** Nucleotide sequence of the region containing *dam* promoters P1 and P2. Nucleotides are numbered relative to the *Bgl*II site upstream of the *aroB* gene. The endpoints of the *Bal*31 deletions carried on plasmids pALO205–213 are indicated, as are the mapped transcript start points for promoters P1 and P2 (see below). The amino acid sequence for the *AroK* protein is shown in the three-letter code. Nucleotides 2002–2280 are identical to nucleotides 9–287 of the published *aroB* sequence (Millar and Coggins, 1986). These sequence data will appear in the EMBL/GenBank/DBJ Nucleotide Sequence Data Libraries under the accession number M76389.

The organization of the five promoters makes it tempting to speculate that promoters P1 and P2 serve to ensure a basal level of transcription for the *aroK*, *aroB*, *urf74.3*, and *dam* genes. Promoter P3 may modulate levels of the *urf74.3* and *dam* gene products, whereas promoters P4 and P5 may fine-tune *dam* gene expression. If this is the case, promoters P1 and P2 mainly fulfil the demands for *aroK* and *aroB* gene products.

#### Flow cytometric analysis of *dam* operon mutants

Initiation of DNA replication in a single cell is co-ordinated so that all origins initiate simultaneously. This co-ordination is sensitive to the level of Dam methyltransferase, i.e. too little or too much Dam methyltransferase yields asynchrony (Boye and Løbner-Olesen, 1990). A measure of initiation synchrony in single cells therefore provides a simple way of analysing the effects of different chromosomal mutations in the *dam* operon.

We performed a flow cytometric analysis on a wild-type strain and three derivatives with different mutations in the *dam* operon. The cells were grown exponentially, treated with rifampicin (Rif) to block new initiations of replication, and with cephalixin to block cell division. After termination of ongoing DNA synthesis the cells were stained for DNA content. Cells performing synchronous initiations contain  $2^n$  chromosomes after treatment with Rif and cephalixin, whereas asynchronous initiations lead to cells with chromosomal numbers different from  $2^n$  after drug treatment (Skarstad *et al.*, 1986).

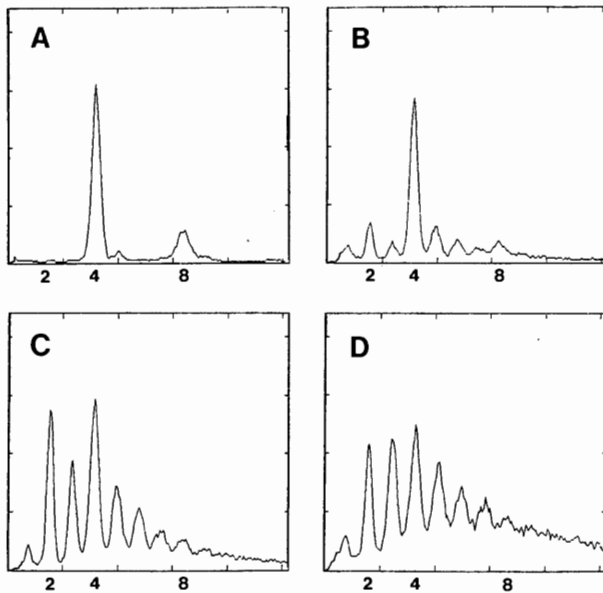
Cells of the wild-type LJ24 contain four or eight fully replicated chromosomes after treatment with Rif and cephalixin (Fig. 8A) and thus initiate chromosome replication in a perfectly synchronous manner.

Strain ALO939 carries an insertion of the *cat* gene from plasmid pACYC184 in *aroK* between P2 and P3. This insertion reduces transcription of the *dam* gene to approximately 30% of the control cells without the insertion (data not shown). Initiation of DNA replication in strain ALO939 is slightly asynchronous, as revealed by the presence of cells containing irregular numbers of chromosomes after treatment with Rif and cephalixin (Fig. 8B).

When a mini-Tn10 is inserted into the *urf74.3* gene between P3 and P4 (strain ALO863), transcription of the *dam* gene is reduced to approximately 10% of the control cells (not shown). Initiation of replication in this strain is almost totally asynchronous; however major peaks still appear, corresponding to two and four chromosomes (Fig. 8C).

Cells containing the *dam-16::Km* allele (Parker and Marinus, 1988) contain no Dam methyltransferase, and consequently initiation of DNA replication is totally random (Fig. 8D).





**Fig. 8.** Single-parameter DNA histograms of *dam* operon mutants. Single-parameter DNA histograms of strains LJ24 (wild type, A); ALO939 (*aroK::cat*, B); ALO863 (*urf74.3::miniTn10*, C); and ALO452 (*dam-16::Km<sup>R</sup>*, D). Cells were grown at 37°C and treated with rifampicin and cephalexin before analysis by flow cytometry. Doubling times for cultures of the four strains were 33, 39, 33 and 34 min, respectively. The positions of cells containing two, four, and eight fully replicated chromosomes are indicated.

*dam::lacZ* transcriptional and translational fusions (Table 1). This indicates that the *dam* transcripts are poorly translated, and in agreement with this we have been unable to identify a good ribosome-binding site preceding *dam*.

The amount of  $\beta$ -galactosidase expressed from either plasmid was the same in wild-type and *dam* mutant strains (Table 1), indicating that the level of Dam methylase is not controlled through an autoregulatory loop. *dam* gene expression is also unchanged in *recA* bacteria (Table 1). In two *lexA* mutant strains a reduction in *dam* gene expression was observed. The SOS system cannot be turned on in the *lexA3* mutant strain, whereas it is constitutively turned on in the *lexA51* strain (Walker, 1987). Since *dam* gene expression was the same in these two strains (Table 1), *dam* does not belong to the group of SOS-regulated genes.

The location of the *dam* gene downstream from the *aroB* gene suggested the possibility that both genes are co-transcribed and, like other *aro* genes, are negatively regulated by the *tyrR* gene product (Pittard, 1987). However, this was not the case (Table 1), in agreement with the observation that the  $\beta$ -galactosidase activity of plasmid pALO163 was unaffected by the presence of amino acids in the medium (data not shown).

It has been reported that the *dam* gene is negatively regulated by the DnaA protein (Jonczyk *et al.*, 1989). We

therefore measured the  $\beta$ -galactosidase activity of pALO163 and pALO219 in strain ALO454, which carries the *dnaA46* allele encoding a temperature-sensitive DnaA protein. Since the *dnaA46* mutation is conditionally lethal, the activities of plasmids pALO163 and pALO219 were determined at the permissive temperature. Even at the permissive temperature, the DnaA46 protein is only partially active and derepresses its own synthesis (Atlung *et al.*, 1985). In agreement with Jonczyk *et al.* (1989), we did observe a slight increase in expression of the *dam* gene in a *dnaA46* mutant strain at the permissive temperature (Table 1). However no further increase in expression of the *dam* gene in this strain was observed upon incubation at the non-permissive temperature (data not shown). This is in contrast to the situation in well-characterized DnaA-regulated genes such as *dnaA* and *mioC* (Atlung *et al.*, 1985; Løbner-Olesen *et al.*, 1987), in which a three- to fourfold derepression is observed at the non-permissive temperature. Therefore it is unlikely that the overall regulation of the *dam* gene is affected by the DnaA protein. The apparent discrepancy between our conclusion and the one reached previously (Jonczyk *et al.*, 1989) may be explained by the fact that we have studied the activity of promoters P1–P5, whereas Jonczyk *et al.* (1989) studied a DNA fragment containing P4 and P5 only, which contribute little to total *dam* expression.

**Table 1.** Activity of *dam-lacZ* fusion plasmids.

Host strain	Relevant genotype	$\beta$ -Galactosidase Activity	
		pALO163	pALO219
LJ24	Wild type	150	3
ALO452	<i>dam16::Km<sup>R</sup></i>	129	2
ALO454	<i>dnaA46</i>	245	4
AB1157	Wild type	148	4
GM2927	<i>dam13::Tn9</i>	197	5
GM2932	<i>muth34</i>	205	3
GM2972	<i>dam13::Tn9</i> , <i>muth34</i>	175	4
DM2251	Wild type	192	4
DE192	<i>lexA51</i>	121	3
DE407	<i>lexA3</i>	133	3
MC1000	Wild type	150	3
NF1815	<i>recA1</i>	157	3
S20	<i>rho-213</i>	ND	4
DRS372	Wild type	ND	6
DRS361	<i>himA</i> ( $\Delta$ SmaI), <i>himD</i> ( $\Delta$ 3)::Cam <sup>R</sup>	ND	7
ALO865	Wild type	239	ND
ALO866	<i>tyrR366</i>	151	ND

Cells were grown exponentially in minimal medium supplemented with glucose, casamino acids, and ampicillin at 37°C, except strain ALO454 which was grown at 30°C. Strains ALO865 and ALO866 were grown in a minimal medium supplemented with glucose, threonine, leucine, aromatic amino acids/vitamins and ampicillin. The  $\beta$ -galactosidase activities of the individual cultures were determined as described in the *Experimental procedures*. ND: not determined.

*dam* gene expression was also tested in a *mutH* strain since the MutH protein is known to bind to hemimethylated and unmethylated DNA (Welsh *et al.*, 1987). No significant difference in activity was detected between *mutH*, *dam mutH*, and wild-type strains (Table 1).

Table 1 also shows that mutations affecting the RNA polymerase termination factor Rho or integration host factor (*himA*, *himD*) do not affect *dam* gene expression.

Thus we can conclude that the products of genes *dam*, *mutH*, *recA*, *lexA*, *rho*, *himA*, *himD*, and *tyrD* are not involved in regulating *dam* gene expression in *E. coli*, either at the transcriptional or the translational level.

## Conclusions

The results obtained from *lac* fusions and primer extension analysis indicate that five promoters contribute to transcription across the *dam* gene and that the major promoter is located 3.2 kb upstream of the translation start codon. Further support for the existence of multiple promoters was obtained by flow cytometric analysis of strains harbouring drug-resistance sequences in the *aroK* or *urf74.3* chromosomal genes. The results indicated that transcription initiation upstream of these genes is important for proper synchronous initiation of chromosome replication and, by extension, for transcription of the *dam* gene.

Alignment of the five promoter sequences failed to show any homology to each other, except for the regions that are thought to interact with the  $\sigma^{70}$  subunit of RNA polymerase. This suggests that the promoters are not regulated by a common mechanism. The isolation and characterization of mutant strains with increased or decreased *dam* gene expression may help to elucidate the roles of the various promoters.

## Experimental procedures

### Growth media

Cells were grown in either Luria Broth (LB; Bertani, 1951) or in AB medium (Clark and Maaløe, 1967) supplemented with 0.5  $\mu\text{g ml}^{-1}$  thiamine and 0.2% glucose. Casamino acids were added to a final concentration of 1%. When required, threonine and leucine were added to a final concentration of 50  $\mu\text{g ml}^{-1}$ . A mixture of aromatic amino acids and vitamins contained 1 mM phenylalanine (165  $\mu\text{g ml}^{-1}$ ), 1 mM tyrosine (191  $\mu\text{g ml}^{-1}$ ), 0.5 mM tryptophan (102  $\mu\text{g ml}^{-1}$ ), 0.1 mM *p*-aminobenzoic acid (14  $\mu\text{g ml}^{-1}$ ) and 0.4 mM *p*-hydroxybenzoic acid (138  $\mu\text{g ml}^{-1}$ ) (Pittard and Wallace, 1966). MacConkey agar was obtained from Difco. Ampicillin and tetracycline were added to final concentrations of 50  $\mu\text{g ml}^{-1}$  and 8  $\mu\text{g ml}^{-1}$ , respectively.

### Bacterial strains

All bacterial strains used were derivatives of *E. coli* K-12 and are listed in Table 2.

**Table 2.** Bacterial strains.

Strain	Genotype	Source/Reference
LJ24	<i>supE44</i> , <i>rpsL</i> , <i>rfbD1</i> , <i>thi-1</i> , <i>leuB6</i> , $\Delta$ ( <i>lacIZ</i> ), <i>lacY1</i> , <i>tonA21</i>	Rasmussen <i>et al.</i> (1991)
KL335	<i>lacI3</i> , <i>lacZ608</i>	K. B. Low
MM294	<i>endA1</i> , <i>thi-1</i> , <i>hsdR17</i> , <i>supE44</i>	Rewinski and Marinus (1987)
GM3819	<i>dam-16::Km<sup>R</sup></i>	Parker and Marinus (1988)
TC743	<i>dnaA46</i> , <i>tna::Tn10</i>	T. Atlung
AB2847	<i>aroB354</i> , <i>tsx-354</i>	CGSC <sup>1</sup>
AB1157	F <sup>-</sup> , <i>thr-1</i> , <i>ara-14</i> , $\Delta$ ( <i>gpt-proA</i> )62, <i>lacY1</i> , <i>tsx-33</i> , <i>supE44</i> , <i>galK2</i> , <i>hisG4</i> , <i>rfbD1</i> , <i>mgl-51</i> , <i>rpsL31</i> , <i>kdgK51</i> , <i>xyl-5</i> , <i>mtl-1</i> , <i>argE3</i> , <i>thi-1</i>	E. A. Adelberg
GM2927	<i>dam-13::Tn9<sup>a</sup></i>	Marinus <i>et al.</i> (1983)
GM2932	<i>mutH34<sup>a</sup></i>	Lab. stock
GM2972	<i>dam-13::Tn9</i> , <i>mutH34<sup>a</sup></i>	Lab. stock
MC1000	<i>thi-1</i> , $\Delta$ ( <i>ara-leu</i> )7679, <i>araD139</i> , $\Delta$ ( <i>lacIZY</i> ), <i>galU</i> , <i>galK</i> , <i>rpsL</i>	Casadaban and Cohen (1980)
NF1815	<i>recA1<sup>b</sup></i>	N. Fill
S20	<i>rho-213<sup>b</sup></i>	S. Brown
DM2251	$\Delta$ ( <i>lac-pro</i> ) <sub>xiii</sub> , <i>mtl-1</i> , <i>rpsL31</i> , <i>thi-1</i>	Fram <i>et al.</i> (1989)
DE192	<i>lexA51<sup>c</sup></i>	Fram <i>et al.</i> (1989)
DE407	<i>lexA3<sup>c</sup></i>	Fram <i>et al.</i> (1989)
ALO452	<i>dam16::Km<sup>Rd</sup></i>	LJ24 $\times$ P1(GM3819)
ALO454	<i>dnaA46</i> , <i>tna::Tn10<sup>d</sup></i>	LJ24 $\times$ P1(TC743)
ALO863	<i>urf74.3::Mini-Tn10<sup>d</sup></i>	This work
ALO939	<i>aroK::cat<sup>d-g</sup></i>	Løbner-Olesen and Marinus (1992)
DRS372	<i>lacU169</i> , $\Delta$ ( <i>trpEA</i> )2	Denise Roberts
DRS361	<i>himA</i> ( $\Delta$ <i>smal</i> )::Tn10Km, <i>himD</i> ( $\Delta$ 3)::Cam <sup>Re</sup>	Denise Roberts
ALO865	F <sup>-</sup> , <i>thi</i> , <i>thr-1</i> , <i>leuB6</i> , <i>aroL478::Tn10</i> , $\Delta$ <i>lacZM15</i> , <i>supE44</i> , <i>tonA2</i> , <i>gyrA379</i> , <i>hsdR</i> , $\lambda$ <sup>+</sup>	J. Pittard
ALO866	F <sup>-</sup> , <i>thi</i> , <i>thr-1</i> , <i>leuB6</i> , <i>aroL516</i> , $\Delta$ <i>lacZM15</i> , <i>tyrR366</i> , <i>zcf-352::Tn10</i> , <i>supE44</i> , <i>tonA2</i> , <i>gyrA379</i> , <i>hsdR</i> , $\lambda$ <sup>+</sup>	J. Pittard

a. In addition to the markers of AB1157.

b. In addition to the markers of MC1000.

c. In addition to the markers of DM2251.

d. In addition to the markers of LJ24.

e. In addition to the markers of DRS372.

f. CGSC: *E. coli* Genetic Stock Centre.

g. Contains plasmid pMS2 (Løbner-Olesen and Marinus, 1992).

### Enzymes

Restriction endonucleases, DNA Polymerase I Klenow fragment, Bal31 nuclease and T4 DNA ligase were obtained from, and used, as recommended by New England Biolabs. AMV reverse transcriptase was purchased from, and used, as recommended by Promega Biotech.

### Plasmid pJEL109

Plasmid pJEL109 (Fig. 1; J. E. L. Larsen, unpublished data) is an R1-derived vector with a copy number of one to two per host chromosome (Scott, 1984). It contains the R1 origin of replication, the *bla* gene of Tn3, and unique cloning sites for the restriction enzymes *Bam*HI, *Cla*I and *Hind*III.

### Construction of *dam-lacZ* fusion plasmids

The *dam-lacZ* transcriptional fusion plasmid, pALO163, was constructed by inserting the promoterless *lacZ* gene carried on a 3 kb *Bam*HI-*Dra*I fragment from plasmid pTL25 (Linn and Ralling, 1985) into the unique *Bam*HI site of plasmid ALO160. Insertion of the same *lacZ*-carrying fragment into plasmid pJEL109 digested with *Bam*HI and *Hind*III resulted in plasmid pALO161, which served as a control for non-specific transcription entering the *lacZ* gene from vector sequences. The *dam-lacZ* translational fusion plasmid pALO219 was constructed by inserting the *Bam*HI fragment carrying *lacZYA* from plasmid pMQ132 (Arraj *et al.*, 1990) into the unique *Bam*HI site of plasmid pALO160. This construction fuses the first 104 amino acids of the Dam protein to amino acid number 9 of the LacZ protein.

### Construction of deletion derivatives of plasmid pALO163

Plasmid pALO163 was digested with *Nru*I and treated with nuclease Bal31 for various times before ligation in the presence of a *Bgl*II linker (New England Biolabs #1066). A selected number of the resulting clones were digested with *Bgl*II and *Hind*III and the *lacZ*-bearing fragments were ligated to plasmid pJEL109 previously digested with *Bam*HI and *Hind*III. This procedure resulted in the formation of plasmids pALO177, pALO179, pALO181, pALO183, pALO184, pALO186, pALO188, pALO190, pALO192, pALO194, pALO196, pALO198, and pALO200 (Fig. 2). Plasmids with Bal31-generated deletions in the opposite direction to those above were obtained by digestion with *Bgl*II and *Bam*HI. This yielded plasmids pALO205, pALO206, pALO207, pALO208, pALO209, pALO210, pALO211, pALO212, pALO213, and pALO214 (Fig. 3). Plasmid pALO201 (Fig. 2) was constructed by inserting the *Bal*-*Hind*III *lacZ* fragment from plasmid pALO163 into pJEL109 digested with *Bam*HI and *Hind*III. Plasmids pALO215, pALO216, and pALO217 (Fig. 4) were made by resection of plasmid pALO163 using the enzymes *Nru*I+*Bam*HI, *Rsr*II+*Bam*HI and *Eco*RI+*Bam*HI, respectively.

### Construction of chromosomal mutations in the *dam* operon

Strain LJ24 containing plasmid pALO163 was mutagenized with mini-Tn10 using the transposable element carried on bacteriophage lambda NK1098 (Way *et al.*, 1984). Plasmid pALO165 carried the mini-Tn10 inserted in the *urf74.3* gene between promoters P3 and P4. The transposon was recombined onto the chromosome using the method of Parker and Marinus (1988), resulting in strain ALO863. The presence of the transposon in the right position on the chromosome was verified by Southern blotting (data not shown).

Strain ALO803 carries the *cat* gene of plasmid pACYC184 inserted in the *aroK* gene between P2 and P3. The detailed construction of this strain is described elsewhere (Løbner-Olesen and Marinus, 1992). This insertion has a polar effect on expression of the *aroB* gene and leads to an Aro<sup>-</sup> phenotype. In order for ALO803 to obtain growth rates comparable with the wild type, this was transformed with the *aroK-aroB*-carrying plasmid, pMS2 (Løbner-Olesen and Marinus, 1992), to yield strain ALO939.

Strain ALO452 was constructed by P1-transducing the *dam16::Km* allele from strain GM3819 into LJ24.

### $\beta$ -galactosidase assay

$\beta$ -galactosidase activity of the different *dam-lacZ* fusion plasmids was assayed in strain LJ24. The growth medium was AB minimal medium supplemented with thiamine, glucose, casamino acids, and ampicillin. Cells were permeabilized by toluene and  $\beta$ -galactosidase units calculated essentially as described by Miller (1972). In those cases where  $\beta$ -galactosidase activity was greater than 500 units, the enzyme was assayed in crude extracts obtained by sonication. Enzyme levels of each strain were determined as an average of five samples taken at different optical densities of the culture.

### Nucleotide sequencing

Nucleotide sequencing was performed essentially as described by Sanger *et al.* (1977) using plasmid DNA prepared from strain MM294 (Rewinski and Marinus, 1987). The sequence obtained was merged with those of Millar and Coggins (1986), Jonczyk *et al.* (1989) and Brooks *et al.* (1983). In this combined sequence, numbering starts at the first base of the *Bgl*II site placing the *aroK* gene at position 1791-2223, the *aroB* gene at position 2450-3536, the *urf74.3* open reading frame at 3628-4908, and *dam* at 5018-5851.

### Preparation of RNA

RNA was prepared from strain LJ24 containing the appropriate plasmid by the cold phenol method described previously (Gerdes *et al.*, 1988).

### Primer extension analysis

Primer extensions were done according to Sambrook *et al.* (1989), and the resulting DNA fragments were separated on 8% polyacrylamide urea gels, next to sequencing reactions of plasmid pALO160 DNA using the same primers. The primers used were MM46 (CTCCAGCGGGTTGGC), MM44 (GCCTTC TTCGCCCTTC), MM55 (GCGGTAAATACGCCTGCGCGG) and MM51 (ACCAGGACATACCACGGC) for promoters P1 to P4, respectively. In the cases where primer-extended products were present as doublets, the longer product was taken to represent the transcriptional start site.

### Flow cytometry

Treatment with antibiotics and flow cytometry was performed essentially according to Boye and Løbner-Olesen (1990) using an 'Argus 100' flow cytometer.

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