JOURNAL OF BACTERIOLOGY, Feb. 2001, p. 1233–1241 Vol. 183, No. 4
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.4.1233–1241.2001
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A Putatively Phase Variable Gene (*dca*) Required for Natural Competence in *Neisseria gonorrhoeae* but Not *Neisseria meningitidis* Is Located within the Division Cell Wall (*dcw*) Gene Cluster

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Received 23 August 2000/Accepted 14 November 2000

A cluster of 18 open reading frames (ORFs), 15 of which are homologous to genes involved in division and cell wall synthesis, has been identified in *Neisseria gonorrhoeae* and *Neisseria meningitidis*. The three additional ORFs, internal to the *dcw* cluster, are not homologous to *dcw*-related genes present in other bacterial species. Analysis of the *N. meningitidis* strain MC58 genome for foreign DNA suggests that these additional ORFs have not been acquired by recent horizontal exchange, indicating that they are a long-standing, integral part of the neisserial *dcw* gene cluster. Reverse transcription-PCR analysis of RNA extracted from *N. gonorrhoeae* strain FA19 confirmed that all three ORFs are transcribed in gonococci. One of these ORFs (*dca*, for division cluster competence associated), located between *murE* and *murF*, was studied in detail and found to be essential for competence in the gonococcal but not in the meningococcal strains tested. Computer analysis predicts that *dca* encodes an inner membrane protein similar to hypothetical proteins produced by other gram-negative bacteria. In some meningococcal strains *dca* is prematurely terminated following a homopolymeric tract of G's, the length of which differs between isolates of *N. meningitidis*, suggesting that *dca* is phase variable in this species. A deletion and insertional mutation was made in the *dca* gene of *N. gonorrhoeae* strain FA19 and *N. meningitidis* strain NMB. This mutation abrogated the ability of the gonococci to be transformed with chromosomal DNA. Thus, we conclude that the *dca*-encoded gene product is an essential competence factor for gonococci.

The proteins encoded by bacterial division and cell wall (*dcw*) gene clusters are essential for viability (16). Significant progress has been made in recent years in understanding the genes involved in cell wall biosynthesis and division (5, 6, 7, 11, 32, 62). Fifteen genes located at 2 min on the *Escherichia coli* chromosome have been identified and are called the *dcw* cluster (4, 64). The genes of this cluster are tightly packed, some overlapping, and are oriented in one direction (4, 64). Although the *dcw* cluster is highly conserved among evolutionarily diverse bacterial species (48), some variations in the clusters have been reported. These variations include the location of some *dcw* genes at separate chromosomal locations, such as *ftsW*, *murF*, and *ddlA* of *Staphylococcus aureus* (48), and the addition of species-specific genes within the *dcw* cluster, such as *spoVD* and *spoVE* of *Bacillus subtilis* (12, 31). In the case of these *B. subtilis* sporulation genes, *spoVD* (12) and *spoVE* (31) have homology to the *dcw* cluster genes *pbpB* and *ftsW*, respectively. Although not essential for vegetative growth, these genes are essential for sporulation (12, 31).

Since the products of the *dcw* genes encode proteins involved in peptidoglycan synthesis and cellular division, it is necessary to use conditionally lethal mutants to study these genes (5, 6, 11, 16, 27). Moreover, since they are typically expressed at low levels, it has been difficult to examine their regulation and to map their promoter elements (13). However, using promoter reporter constructs, a putative promoter (P_{mra}) upstream of the *E. coli dcw* cluster has been identified. This promoter is required for transcription of at least the first nine genes of the cluster (*yabB* to *ftsW*) and may provide the majority of the transcript for the downstream genes (30, 43).

Neisseria gonorrhoeae and *N. meningitidis* are important, human-specific pathogens and are causative agents of gonorrhea and of meningitis and septicemia, respectively. MtrR is a transcriptional regulator, in gonococci, of both the *mtr* operon and the *far* operon, which encode efflux pump proteins (28, 39). While investigating the regulation of the *mtr* efflux system by MtrR in *N. gonorrhoeae*, we performed a search of the gonococcal genome sequence database for the recognition site of MtrR (40) in an attempt to identify other genes that might be subject to its regulation. A potential MtrR-binding sequence was found upstream of an open reading frame (ORF) with homology to *yabB* of *E. coli* (4). A homologue of *yabB* is the first gene of the *dcw* cluster in all of the bacterial species studied to date (4, 13, 20, 38, 48, 64). Since the *dcw* cluster of the pathogenic *Neisseria* spp. had not been previously described at that time, we decided to investigate this gene cluster in the pathogenic *Neisseria* spp. A recent report (21) has described the gonococcal *dcw* cluster to some extent, although two ORFs (*ftsL* and the homologue of NMB0417) were not identified and a hypothetical gene was defined as the most 3′

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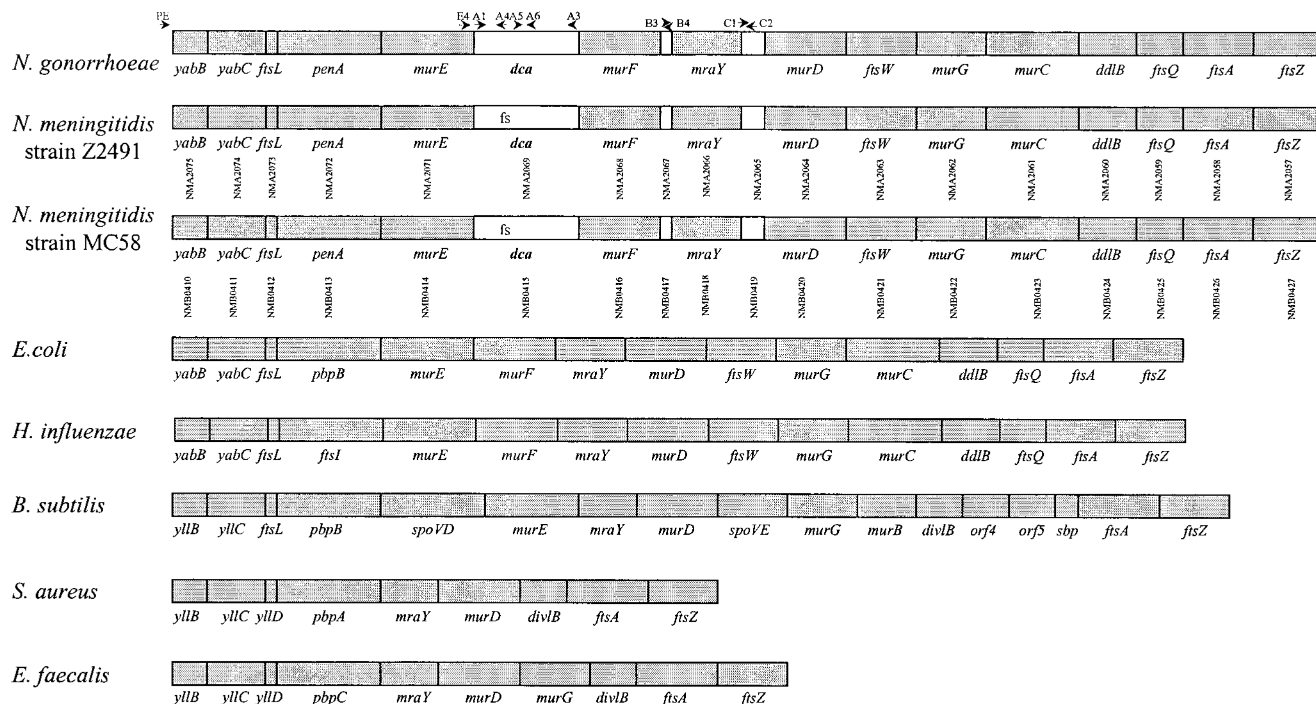

FIG. 1. Comparison of the *dcw* clusters from *N. gonorrhoeae, N. meningitidis* strain Z2491 (46), *N. meningitidis* strain MC58 (59), *E. coli* (4, 64), *H. influenzae* (20), *B. subtilis* (13, 38), *S. aureus*, and *E. faecalis* (48). All genes are transcribed from left to right. Due to variations in nomenclature between the species, functionally conserved genes have different names; the *penA, ftsI*, and *pbp* genes are homologues, as are the *yab* and *yll* genes. The first two ORFs of the *dcw* clusters in this figure are named based on the consistent designations of these ORFs in GenBank. The *spoVD* and *spoVE* genes of *B. subtilis* are homologues of *pbpB* and *ftsW*, respectively, and the proteins they encode have a sporulation-specific function related to that of the PbpB and FtsW function in vegetative growth (12, 31). NMA and NMB annotation numbers are indicated below each ORF (46, 59). White boxes in *N. gonorrhoeae* and *N. meningitidis* represent ORFs not seen in other *dcw* clusters. The NMB0417 and NMB0419 homologues are of unknown function and are therefore not named in *N. gonorrhoeae*. The relative positioning of the frameshift in *dca* in *N. meningitidis* is indicated (fs). The relative positions of the oligonucleotides from Table 1 are shown (arrows). PE, *yabB*PE; E4, *murE*#4; A1, *orfA*#1; A3, *orfA*#3; A4, *orfA*#4; A5; *orfA*#5; A6, *orfA*#6; B3, *dcaB*#3; B4, *dcaB*#4; C1, *dcaC*#1; C2, *dcaC*#2. Apparent overlap is due to the limitations of the figure.

gene of the cluster, rather than the conserved *ftsZ*. We report that while the *dcw* clusters of gonococci and meningococci are broadly similar to those described in other species, they contain three additional ORFs internal to the *dcw* cluster that are not present in the *dcw* clusters of other the bacterial species studied to date. We demonstrate that these ORFs do not have features of recent horizontal acquisition and that they are all transcribed. One of these ORFs, *dca*, has features suggestive of phase variability associated with polymorphisms between strains and is associated with natural competence in the gonococcal strains, although not in the meningococcal strains tested.

MATERIALS AND METHODS

Bacterial strains and growth conditions. We used *N. gonorrhoeae* strains FA19 and FA1090 (kindly provided by P. F. Sparling and J. Cannon, respectively, of the University of North Carolina School of Medicine, Chapel Hill). *N. meningitidis* invasive disease isolate strains (NMB [capsular serogroup B], 0929 [serogroup Y], and 2633 [serogroup Y]) and six commensal *Neisseria* spp. (*N. flavescens, N. lactamica, N. macacae, N. polysaccharea, N. sicca*, and *N. subflava*) were kindly provided by D. Stephens (Emory University School of Medicine, Atlanta, Ga.). A transformant of strain FA19 containing an insertional inactivation and deletion in the *recA* gene was constructed by transformation using pC68a (kindly provided by C. Gibbs and T. Meyer), which contains *recA*::*ermC*. All strains were propagated on GCB agar or in GCB broth (Difco Laboratories, Detroit, Mich.) containing the Kellogg supplements and ferric nitrate (36) at 37°C under 3.8% (vol/vol) CO_2. Growth in liquid media employed nonpiliated, transparent colony variants with growth monitored by determining the optical density at 600 nm. Spontaneous streptomycin-resistant (Str^r) mutants of gonococci were selected by plating 10^9 CFU of strain FA19 onto GCB agar plates containing 100 μg of streptomycin per ml. Str^r mutants of meningococci were made by transformation of strain NMB with Str^r gonococcal chromosomal DNA. Transformants were selected on GCB agar plates containing either 100 μg of streptomycin per ml or 1 μg of erythromycin per ml. All antimicrobial agents were obtained from Sigma Chemical Company (St. Louis, Mo.).

PCR amplification, plasmid preparation, and DNA sequencing. Chromosomal DNA extractions were performed using the method of McAllister and Stephens (41). PCR from chromosomal DNA was performed as described previously (29). Plasmids were purified using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions (QIAGEN, Valencia, Calif.). DNA sequencing of PCR products and plasmids was performed using automated sequencing by the Emory DNA Sequencing Core Facility (Atlanta, Ga.) using a PE Applied Biosystems 377 automated DNA sequencers (Foster City, Calif.). Sequencing was carried out by the modified method of Sanger et al. (50) using BigDye reaction chemistry (ABI PRISM; PE Applied Biosystems, Norwalk, Conn.). PCR products for sequencing of *dca* were generated with combinations of oligonucleotide primers *orfA*#1, *orfA*#3, *orfA*#5, *orfA*#6, and *murE*#4 (Fig. 1 and Table 1). DNAStar was used for nucleotide and amino acid sequence analysis and alignments (8). Protein topology predictions were generated using the algorithm of von Heijne (60, 61), as implemented by TopPredII (10), and PSORT (45).

DNS. A DNA sequence can be considered to consist of a string of dinucleotides. Analysis of the proportions of dinucleotides in large sequences has revealed that species differences are consistently present in genome sequence compositions (34, 35). This approach has been used to identify regions of horizontally transferred DNA in *N. meningitidis* MC58 (59). We used a modification of this

TABLE 1. Oligonucleotides used for PCR and sequencing

Primer	Oligonucleotide sequence (5′-3′)
orfA#1	ATGAAACAATCCGCCCGAATA
orfA#3	CGGCATTTTTTCTGTACGTA
orfA#4	AATCAGTAAAACAACTGAAG
orfA#5	ATCCTTATCCAAACGTGTGCA
orfA#6	ATGCCGGCCTGTTGTTGAATAT
murE#4	GGATAAAGTCGTCGTAACCA
dcaB#3	TGAAAATGAAAAGCCGACG
dcaB#4	TAACGTGCAGGGACTTTC
dcaC#1	CCGCCCTGATTGCCTTGG
dcaC#2	CTTCAGGCGTTGGTCATTGTC
*yabB*PE	CTGTCGATGCTTAATTCGTGTGCGCCGCCGAACAT

methodology that addresses single ORFs (44; J. Mirsky, N. J. Saunders, J. F. Peden, and S. Jarvis, unpublished data). We have used dinucleotide signature analysis (DNS) to evaluate the possibility that the ORFs within the *dcw* cluster were acquired by recent horizontal transfer of DNA.

Nucleotide sequence searches. The Basic Local Alignment Search Tool (BLAST) (1) was used to search publicly available microbial genome sequences and GenBank. The sequences of *yabB, yabC, ftsL, penA, murE, dca, murF*, the homologue of NMB0417, *mraY*, the homologue of NMB0419, *murD, ftsW, murG, murC, ddlB, ftsQ, ftsA*, and *ftsZ* for gonococci were obtained from the *N. gonorrhoeae* Genome Sequencing Project at the University of Oklahoma (http://www.genome.ou.edu/gono.html). The corresponding meningococcal sequences from serogroup A strain Z2491 were obtained from the Sanger Centre. This sequence data was produced by the *N. meningitidis* Sequencing Group at the Sanger Centre and can be obtained online (http://www.sanger.ac.uk/Projects/N_meningitidis/) (46). The meningococcal sequence from serogroup B strain MC58 was produced by The Institute for Genomic Research (http://www.tigr.org/tdb/CMR/gnm/htmls/SplashPage.html) (59). The sequence of *dca* from *N. meningitidis* serogroup C strain FAM18 was produced by the Sanger Centre (http://www.sanger.ac.uk/Projects/N_meningitidis/seroC.shtml). GenBank was accessed through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The sequences of the *Actinobacillus actinomycetemcomitans* HK1651 *dca* homologues were obtained from the University of Oklahoma (http://www.genome.ou.edu/act.html). The sequence of the *Salmonella enterica* serovar Typhi strain CT18 *dca* homologue was obtained from the Sanger Centre (http://www.sanger.ac.uk/Projects/S_typhi/).

Primer extension analysis. Total RNA was prepared using the hot-phenol method (3). Primer extension products were generated from the *yabB*-specific oligonucleotide, *yabB*PE (Table 1), hybridized to 20 μg of total RNA. The DNA sequence was generated using the same oligonucleotide as a primer and therefore corresponds to that of the mRNA (28).

RT-PCR. RNA was prepared as for primer extension analysis. cDNA was generated from 1 μg of RNA reverse transcribed with SuperScript II RNase H^- reverse transcriptase (Gibco-BRL, Grand Island, N.Y.) and ORF-specific oligonucleotide primers *orfA*#4, *dcaB*#4, and *dcaC*#2 (Table 1). The cDNA was PCR amplified with oligonucleotide primer combinations of *orfA*#4 and *orfA*#1, *dcaB*#4, and *dcaB*#3 and *dcaC*#2 and *dcaC*#1 (Fig. 1 and Table 1). In order to detect contaminating DNA in the RNA preparation, control reverse transcription-PCR (RT-PCR) reactions were conducted in the absence of reverse transcriptase.

Southern blotting. A *dca*-specific probe of 1.6 kb was generated by PCR amplification of FA19 chromosomal DNA with *orfA*#1 and *orfA*#3 (Table 1) and labeled using the Boehringer-Mannheim Non-radioactive DNA Genius 2 Labeling Kit according to the manufacturer's instructions (Indianapolis, Ind.). Three micrograms of chromosomal DNA, determined using a Beckman EU65 spectrophotomer (Fullerton, Calif.) at A_{260}, was digested with *Cla*I, separated by 0.6% (wt/vol) agarose gel electrophoresis, and blotted to a 0.45-μM (pore-size) MagnaGraph nylon transfer membrane (Micron Separations, Inc., Westboro, Mass.). Hybridization, washes, and detection were performed as described in the Genius System User's Guide for Membrane Hybridization using anti-digoxigenin alkaline phosphatase conjugate and CSPD (Boehringer Mannheim).

***dca*-inactivation and transformation.** *dca* was PCR-amplified from genomic DNA of strain FA19 using gene-specific primers (*orfA*#1 and *orfA*#3) and inserted into the pCR 2.1-TOPO vector using the TOPO TA Cloning Kit according to manufacturer instructions (Invitrogen, Carlsbad, Calif.). *dca* was excised with *Eco*RI and cloned into pUC19 (New England Biolabs, Beverly, Mass.). For construction of pLS1, the nonpolar *aphA-3* cassette (42) was inserted into the *Nsi*I site 1,319 bp into *dca*, disrupting the gene at the 3′ end. To create pLS3, a PCR product of *dca* was created that would include 349 bp of DNA upstream of the *Bss*HII site, 62 bp within *dca* using primers *murE*#4 and *orfA*#3. This product was then inserted into the pCR 2.1-TOPO vector, excised with *Eco*RI, and inserted into pUC19, as for pLS1. This construct was then cut with *Bss*HII and *Nsi*I, deleting 1,257 bp from *dca*, and the *aphA-3* cassette was inserted. Restriction endonucleases were obtained from New England Biolabs (Beverly, Mass.). The pLS1 and pLS3 constructs were used as templates for PCR. Piliated gonococci (strain FA19) were transformed with PCR products as described by Gunn and Stein (26). Kanamycin-resistant (Km^r) transformants were selected using 50 μg of kanamycin (Sigma) per ml. Sequencing of the representative transformants confirmed the insertion of the *aphA-3* cassette in strains LS1 and LS3 and the deletion of 1,257 bp from *dca* and that this transformation produced no changes in the upstream *murE* gene in strain LS3 (data not presented). RT-PCR studies showed no detectable polar effect of either the *aphA-3* cassette or the deletion on the transcription of the downstream *murF* (data not presented). These transformants, along with strains FA19 and FA19 *recA*::*ermC*, were tested for natural competence by the method of Gunn and Stein (26) and by the agar overlay procedure of Sparling et al. (55) using chromosomal DNA containing the Str^r marker.

Accession numbers. The GenBank accession numbers for *dca* are as follows: *N. gonorrhoeae* strain FA19, AF195057; *N. meningitidis* strain NMB, AF195058; *N. meningitidis* strain 2633, AF195059; and *N. meningitidis* strain 0929, AF195060.

RESULTS

Identification and organization of the *dcw* gene cluster in gonococci and meningococci. The *dcw* gene cluster of *N. gonorrhoeae* was originally identified in a search of the FA1090 genome sequence database for sites with homology to the MtrR regulator consensus binding sequence (40). One putative MtrR-binding site (18 of 31 bases identical) is located 883 bp 5′ of an ORF predicted to encode a protein with homology to the *yabB*-encoded protein of *E. coli* (32.9% amino acid similarity over the whole protein) (4). Subsequent experiments failed to demonstrate specific binding of MtrR to this site (data not presented), suggesting either that MtrR does not bind to this site or that additional cofactors or sequence determinants are required for its binding. However, the DNA sequence 3′ of the putative *yabB* homologue includes an additional 17 tightly packed ORFs orientated in the same direction (Fig. 1), ending with *ftsZ*, usually ascribed as the most 3′ gene of the *dcw* cluster (48). Of the 18 ORFs in this cluster, 15 encode putative proteins with homology to those encoded by *dcw* genes of other bacterial species (Fig. 1). These have between 28.4 and 55.3% amino acid similarity with the corresponding *E. coli* gene products, except for *ftsL* (17% amino acid similarity). This is consistent with *ftsL* frequently being the least-conserved *dcw* gene between different species. For example, although only 16% amino acid identity exists between the *E. coli* and *B. subtilis* genes, Daniel et al. (14) recently named the *B. subtilis* gene *ftsL* on the basis of its position in the *dcw* cluster, the ORF length, and the predicted protein features. Using this approach and the homologies to *E. coli* proteins, we were able to ascribe gene identifications to 15 of the 18 genes in the pathogenic *Neisseria* spp. Of the genes in this cluster, only *penA*, encoding penicillin-binding protein 2, and *ftsZ*, encoding cell division protein FtsZ, have been previously investigated in the *Neisseria* spp. (49, 56, 57). These ascribed identifications are consistent with the combined annotations of the recently published *N. meningitidis* genome sequences, including the annotation of NMB0412 as *ftsL* (46, 59). Recently, Francis et al. (21) published a description of the *dcw* cluster of *N. gonorrhoeae*, but our interpretation differs from theirs in several

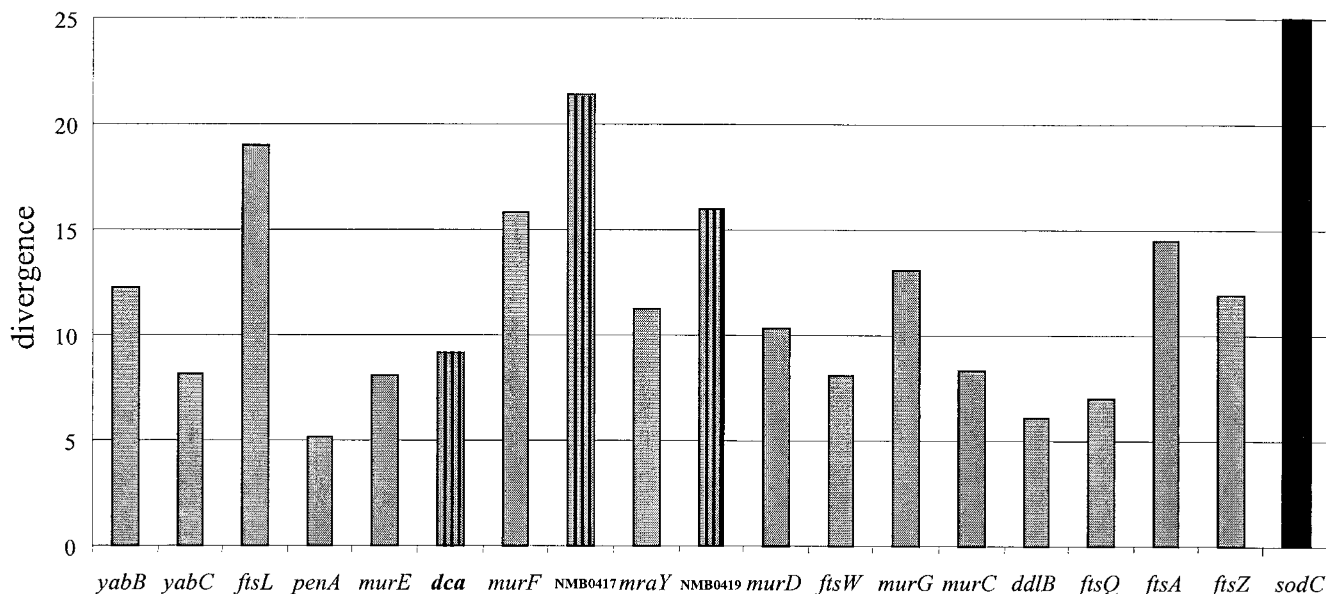

FIG. 2. Dinucleotide signature of the genes within the *dcw* cluster. The figure shows the divergence of the dinucleotide signature for each gene within the *dcw* cluster in *N. meningitidis* strain MC58 from the mean value determined for the complete genome sequence (59) using the method of Mirsky (44). Genes which have been recently horizontally transferred tend to have a divergence of greater than 16% using this type of analysis in this genome (N. J. Saunders, unpublished data), once elevations due to sampling in short ORFs has been accounted for. For example, the *sodC* gene, which is believed to have been horizontally acquired from or via *H. influenzae* (37), has a dinucleotide signature divergence from the mean of 25% (black bar). Striped bars indicate the three additional *dcw* genes: *dca*, NMB0417, and NMB0419.

respects, including the definition of the 3′ end of the cluster as the conserved *ftsZ* rather than an undefined ORF, the number of genes in the cluster as 18 rather than 17, the identification of *ftsL*, and the identification of an additional novel ORF within the cluster, the homologue of NMB0417. Additionally, the present work provides a comparative analysis of the *dcw* cluster from the three sequenced pathogenic *Neisseria* spp. strains, including *N. meningitidis* strains MC58 and Z2491 (Fig. 1).

Using PCR with combinations of oligonucleotide primers that anneal within each of the different ORFs predicted from the FA1090 genome sequence database, we confirmed the presence of a similar *dcw* cluster in the same sequence location in the clonally unrelated gonococcal strain FA19. A search of the two meningococcal genome sequences (strains Z2491 and MC58) revealed that meningococci contain a similar *dcw* gene cluster (Fig. 1) that also includes the three additional ORFs (46, 59). The homologues of these ORFs are designated NMB0415, NMB0417, and NMB0419 in the *N. meningitidis* genome sequence annotation (59). Once we established (see below) that the NMB0415 homologue was involved in competence for transformation, we named it *dca* (division cluster competence associated). The remaining two ORFs have not been named and will be referred to on the basis of their homology with NMB0417 and NMB0419.

A promoter, P_{mra}, located 3′ of *yabB* in *E. coli* has been described that controls transcription of *yabB* to *ftsZ* and is essential for transcription of the first nine *dcw* cluster genes (30, 43). Primer extension analysis was used to seek a similarly located promoter 5′ of the putative gonococcal *dcw* cluster in strain FA19. An extension product ending in the region of a T residue located 68 bp 5′ of *yabB* was detected. Analysis of the DNA sequence in this region revealed candidate −10 (TATAGT) and −35 (TTGACC) regions, separated by 17 bp, generating an optimally spaced σ^{70} consensus binding site located 74 bp 5′ of *yabB* (data not presented). The equivalent putative promoter regions in *N. meningitidis* strains Z2491 and MC58 are identical. These results suggest that the neisserial *dcw* cluster has a promoter, similar to that seen in *E. coli*, 5′ of *yabB*. Other putative and identified promoters are likely to contribute to the transcription of genes internal to the *dcw* cluster (21; L. A. S. Snyder, unpublished data). Francis et al (21) identified six promoters at the 3′ end of the cluster that would contribute to *ftsQAZ* transcription: PQ1 within *ddlB*, PA1 and PA2 within *ftsQ*, and PZ1, PZ2, and PZ3 between *ftsA* and *ftsZ*. In order to learn whether *dca* and the NMB0417 and NMB0419 homologues are transcribed in gonococci, total RNA from strain FA19 was used in RT-PCR reactions that included ORF-specific primers. RT-PCR products were obtained for each ORF (data not presented), indicating that all three were transcriptionally active.

Sequence composition of the additional neisserial ORFs within the *dcw* gene cluster. The pathogenic *Neisseria* spp. are naturally transformable, and this can lead to the acquisition of genes from both related and unrelated bacterial species (51). Given that the pathogenic *Neisseria* spp. examined contain ORFs within the *dcw* cluster that are not present in other bacterial species, we sought to investigate the evolutionary history of these genes by determining whether they have features of recent horizontal acquisition. The *dcw* cluster of *N. meningitidis* strain MC58 was analyzed using a modification of DNS that can assess single ORFs (44; Mirsky et al. unpublished) (Fig. 2). This analysis failed to detect evidence of recent

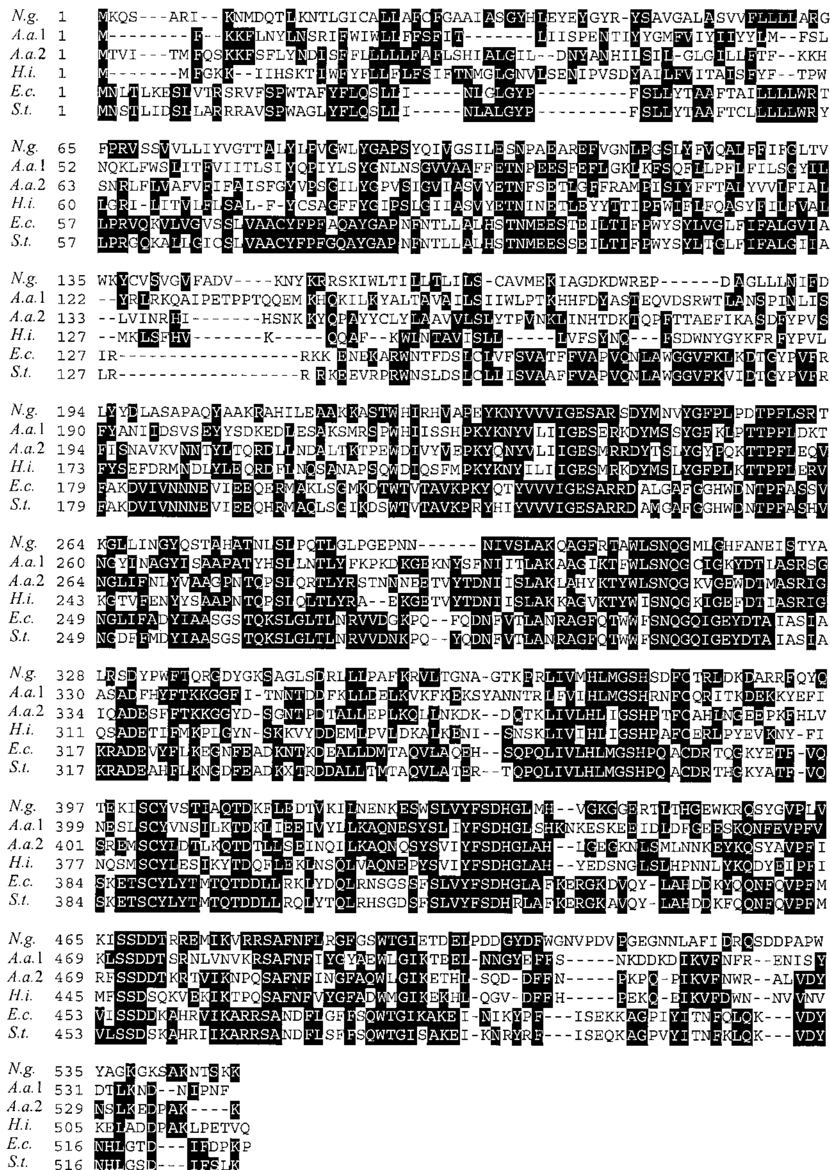

FIG 3. *dca* homologues. Alignment of the predicted amino acid sequence of *dca* from *N. gonorrhoeae* FA19 (*N.g.*) with hypothetical proteins from *A. actinomycetemcometans (A.a.* 1 and *A.a.* 2), *H. influenzae* HI1005 (*H.i.*) (20), *E. coli ybiP* (*E.c.*) (4), and serovar Typhi (*S.t.*). Identical residues are indicated by black boxes.

interspecies horizontal acquisition of any of the genes in the *dcw* cluster, including the additional ORFs. The most atypical of the ORFs are *murF* and *ftsA*, but the degree of divergence is less than that normally associated with horizontally transferred genes in this genome, such as *sodC* (37) (Fig. 2). The short nature of *ftsL* (264 bp) and NMB0417 (177 bp) is sufficient to account for their elevated values in this analysis due to a sampling effect, and the moderate elevation of the NMB0419 homologue (381 bp) can be accounted for on a similar basis.

dcw **cluster-associated ORF** ***dca*****.** We selected *dca*, which is located between *murE* and *murF* in both gonococci and meningococci, for further investigation on the basis of its homology to hypothetical proteins present in other gram-negative species and the presence of a homopolymeric tract suggestive of phase variation (see below). The sequence of *dca* (1,647 bp) from gonococcal strain FA19 was determined. *dca* encodes a predicted protein of 548 amino acids with five transmembrane domains located within the *N*-terminal region between residues 16 and 174 and an *N*-terminal signal sequence of 35 amino acids. Similar hypothetical proteins are present in other microbial genome sequences, but they appear to be restricted to gram-negative bacteria. Alignment of the gonococcal sequence with related proteins of *A. actinomycetemcomitans, E. coli (ybiP), H. influenzae* (HI1005), and serovar Typhi are shown in Fig. 3. Protein structural predictions, obtained by using TopPredII, suggest that these homologues have a structure and conformation similar to that of the predicted *dca* product.

				Dca length
FA1090	417	ATCTGTGGGGG-TATTTGCTGA	437	548 aa
FA19	417	ATCTGTGGGGG-TATTTGCTGA	437	548 aa
Z2491	417	ATCGGGGGGGGGTATTTGCTGA	438	145 aa
MC58	417	ATCGGGGGGGGGTATTTGCTGA	438	145 aa
NMB	417	ATCGGGGGGGGGTATTTGCTGA	438	145 aa
2633	417	ATCGGGGGGGG-TATTTGCTGA	437	548 aa
0929	417	ATCGGGGGGGG-TATTTGCTGA	437	548 aa

FIG. 4. Alignment of the repeat region predicted to mediate phase variation within *dca* from the gonococcal strains FA1090 and FA19 and the meningococcal strains Z2491, MC58, NMB, 2633, and 0929. The frameshift caused by the presence of nine guanines, as in Z2491, MC58, and NMB, brings the underlined termination codon in frame, resulting in a premature termination of the ORF. aa, amino acids.

Perrin et al. (47) recently identified regions of the chromosome of strains Z2491 and FA1090 that are specific to the pathogenic *Neisseria* spp. and are not present in *N. lactamica*, a nonpathogenic commensal organism, which includes a region with homology to HI1005 of *H. influenzae*. As shown in Fig. 3, Dca is homologous to the *H. influenzae* ORF HI1005. In order to extend this observation from the single commensal species to more nonpathogenic species and other strains of the pathogenic species, a Southern blot of *Cla*I-digested DNA from six gonococcal strains, three meningococcal strains, and six commensal *Neisseria* strains was probed with a PCR product containing only *dca*. While the pathogenic *Neisseria* spp. produced hybridizing bands (12 kb for all gonococcal strains, 6.6 kb for meningococcal strains NMB and 2633, and 3.2 kb for meningococcal strain 0929) for the *dca*-containing *Cla*I fragment, no hybridization bands were observed for the nonpathogenic commensal strains (data not presented). These results, which include *N. lactamica*, are consistent with those of Perrin et al. and expand the repertoire of *Neisseria* spp. without a detectable *dca* to include *N. flavescens, N. macacae, N. polysaccharea, N. sicca*, and *N. subflava*.

Analysis of the *dca* sequence from the meningococcal strains Z2491 and MC58 predicted that they encode a protein of only 145 amino acids in length, compared to the predicted 548-amino-acid gonococcal protein. These shorter ORFs are a consequence of premature termination following a homopolymeric tract (HPT) of nine G's located 420 bp through the ORF. The sequence in the equivalent location, including polymorphisms, in *N. gonorrhoeae* is eight bases, which is associated with the presence of the full length ORF (Fig. 4). This suggests the potential for alteration in the expression of *dca* mediated by variation in the length of this HPT (52). In *N. gonorrhoeae* this sequence includes polymorphisms (changes of the first and third bases to T's) which reduce the length of the poly(G) repeat to five bases (Fig. 4). When repeats of this shorter length are present in the phase-variable *lgt* LPS biosynthesis genes in *N. meningitidis* they are relatively stable (33). The presence of the shorter (G)5 repeat element in strains FA19 and FA1090 suggests that this gene would also be stable in these strains. Following identification of the HPT, we investigated this sequence in clinical isolates of *N. meningitidis*. We sequenced the *dca* region from an additional capsular serogroup B isolate strain (NMB) and two serogroup Y invasive disease isolate strains (2633 and 0929). NMB contained a (G)9 HPT, and the serogroup Y strains each contained a (G)8 HPT. Preliminary reads from the sequencing project on the serogroup C meningococcus strain FAM18 indicate that this strain also contains a (G)8 HPT. This suggests that the sequenced *dca* genes from these capsular group A and B strains encode a frame-shifted Dca protein, while the serogroup Y and C strains and *N. gonorrhoeae* strains encode a full-length protein (Fig. 4). These observations are consistent with Dca being a phase-variable protein in which the homopolymeric tract acts as a translational switch.

Insertional-inactivation of *dca*. Based on its location within the *dcw* gene cluster, we hypothesized that the *dca*-encoded protein might participate in the processes of cell division and/or cell wall biosynthesis. In order to investigate this possibility, we constructed two separate nonpolar mutations within *dca* using the Km^r-containing *aphA-3* cassette and then transformed them into their original chromosomal locations in strain FA19. Transformants of strain FA19 were readily obtained with the DNA sequence generated from pLS1 and pLS3, indicating that *dca* is not an essential gene. The transformant generated with the pLS1 sequence contains the *aphA-3* cassette inserted into the *Nsi*I site 1,319 bp into *dca*, disrupting the gene at the 3′ end. The transformant generated with the sequence from pLS3 has 1,257 bp deleted from *dca* (between the *Bss*HII and *Nsi*I sites), leaving only 62 bp of *dca* before the insertion of the *aphA-3* cassette. PCR amplification and sequencing (data not presented) was used to confirm that the *aphA-3* cassette had been inserted into *dca* at the predicted sites within the transformants, which were termed LS1 and LS3, respectively. In transformant LS3, 1,257 bp were deleted from *dca*. This deletion and replacement with the nonpolar *aphA-3* cassette had no detectable effect on transcription of the downstream *murF* gene as determined by RT-PCR (data not presented).

Strains LS1 and LS3 were compared to the parental strain FA19 in a number of experiments to identify the functional consequence(s) of *dca* inactivation. These mutations did not alter gonococcal susceptibility to hydrophobic or hydrophilic antibiotics, or nonionic detergents, as tested by using penicillin, erythromycin, and Triton X-100 (data not presented). Repeated experiments also failed to show any reproducible differences in growth rates between strains FA19, LS1, and LS3 in liquid media as judged by CFU and optical-density analyses (data not presented). We also monitored the rate and extent of [^{3}-H]glucosamine uptake into peptidoglycan in growing cultures of FA19 and LS3 by the method of Dougherty (17) but found no reproducible differences (data not presented). Moreover, the rate and extent of peptidoglycan turnover, as determined by the method of Dillard and Seifert (15) using prelabeled cultures, appeared to be similar in the parent and mutant strains in repeated experiments (data not presented).

Natural competence for transformation of *dca* mutants. In the course of investigating the phenotypes of the mutants, an apparent deficiency in transformation was noted in strain LS3. To determine whether strains LS1 and LS3 were deficient in transformation, we selected piliated variants (on the basis of colony morphology with the presence of pili confirmed by electron microscopy) and examined their capacity to be transformed with chromosomal DNA, using piliated variants of parental strain FA19 and FA19 *recA*::*ermC* as controls. As shown in Table 2, we found that strains FA19 and LS1 were readily transformable (frequencies of 10^{-3}) using a chromo-

TABLE 2. Dca is required for transformation of gonococci but not meningococci

Strain	Str[r] CFU ml^{-1}/total CFU ml^{-1}[a]
N. gonorrhoeae	
FA19	8.3×10^{-3}
LS1	3.5×10^{-3}
LS3	$<7.8 \times 10^{-8}$
FA19 *recA*::*ermC*	$<2.6 \times 10^{-8}$
N. meningitidis	
NMB	1.8×10^{-3}
NMLS3	1.8×10^{-3}

[a] Three independent experiments, each done with 0.5 μg of DNA, produced similar results.

somal DNA marker (streptomycin resistance), while LS3 was reproducibly nontransformable (frequency of $<10^{-8}$). In parallel experiments, a *recA*::*ermC* insertional mutant of strain FA19 was also nontransformable (frequency of $<10^{-8}$) (Table 2). The association between the transformation defect in strain LS3 and the *dca*::*aphA-3* mutation was confirmed by analyzing three independent *dca*::*aphA-3* transformants of strain FA19 obtained during the original construction of LS3. All three transformants displayed the same transformation defect exhibited by the first LS3 strain (frequency of $<10^{-8}$). We also examined the capacity of these piliated strains to be transformed with donor DNA using the agar overlay transformation procedure of Sparling et al. (55). However, regardless of the liquid or plate transformation conditions, transformants of strains LS3 or FA19 *recA*::*ermC* could not be recovered (frequencies of $<10^{-8}$).

We next examined the consequence of *dca* inactivation on the capacity of meningococci to be transformed with meningococcal chromosomal DNA harboring the Str marker. For this purpose we used strain NMB, which encodes a truncated (145-amino-acid) Dca protein due to the presence of a phase-off HPT tract of nine G residues (Fig. 4). A *dca*::*aphA-3* PCR product from gonococcal strain LS3 was used to transform NMB for Km[r], and the intended mutation in *dca* was confirmed by PCR and sequencing. A representative transformant (NMLS3), along with the parental strain, was tested for its ability to be naturally transformed by meningococcal (strain NMB) chromosomal DNA marked with Str[r] using the transformation method of Gunn and Stein (26). Both *N. meningitidis* strain NMB and the mutant strain NMLS3 were readily transformed to Str[r] (frequencies of 10^{-3}; Table 2).

DISCUSSION

Horizontal gene transfer is thought to be an important component of bacterial adaptation and evolution. The *Neisseria* spp. are naturally competent for transformation (9, 54), meaning that they are capable of taking up external DNA and incorporating it into their chromosome by homologous recombination. Natural transformation predominantly occurs within populations of cells that are of the same or closely related species, but it is probably also involved in the acquisition of new genes from unrelated species. In *Neisseria* spp., species-specific targeting is largely mediated by the presence of a 10-bp uptake signal sequence (GCCGTCTGAA) which is frequently located 3′ of ORFs, (18, 25). Transformation facilitates the generation of mosaic genes through recombination between existing alleles, new gene acquisition, and possibly repair of genes whose function has been lost by mutation. Recognized examples of this process in *Neisseria* spp. include (i) the *argF* gene of *N. meningitidis*, which includes sections derived from a species similar to *N. cinerea* and from an additional unidentified source (65); (ii) the generation of Opa variants within a population (2); (iii) the acquisition of penicillin resistance through the incorporation of sequences from *N. flavescens* and *N. cinerea* (56, 57, 58); and (iv) the acquisition of foreign genes from other species (37). This is also likely to be the primary route by which silent pilus cassettes are provided for antigenic variation by recombination with the expressed *pilE* gene (53).

Natural transformation is an integral part of neisserial biology and involves several genes. In addition to the requirement for the expressions of pili (22, 54), other proteins have been shown to have a role in natural transformation in the *Neisseria* spp. These include Tpc, mutants of which form tetracocci deficient in epithelial cell invasion and competence (23); ComA, an inner membrane protein which may be involved in translocation of DNA into the cytoplasm (19); ComL, a peptidoglycan-bound lipoprotein, mutants of which are significantly decreased in transformation (24); and PilT, which is involved in twitching motility and DNA uptake (63). Our results indicate that *dca* is also essential for transformation in gonococci. The fact that LS1 retained its competence indicates that the 3′ end of *dca* is not involved in this function. In separate experiments we also evaluated the capacity of strain FA1090 and FA1090 *dca*::*aphA*-3 (obtained using donor DNA from LS3) to be transformed with gonococcal DNA. Using an erythromycin resistance marker from strain KH15 (as FA19 but *mtrR*-171 [28]), we found that *dca* was also required for transformation in strain FA1090 (frequency of $<10^{-8}$).

The ability of the meningococcal strain NMB to retain its competence suggests that *N. meningitidis* transformation occurs through an alternate and/or additional pathway. Alternatively, there may be functional redundancy with the *dca*-encoded product or the *dca*-associated system. Although no homologues to *dca* were found in either the *N. meningitidis* strain Z2491 or MC58 genomes in addition to *dca* itself and only one band appears on Southern blots probed for *dca*, this does not rule out the possibility of more diverse proteins with similar functions. Similar inactivation of *dca* in serogroup Y meningococcal strain 0929, which has a (G)8 HPT (Fig. 4), and thus a full-length *dca* ORF, also did not affect competence (frequency of 10^{-3}). The fact that this ORF is retained in the chromosome and is putatively phase variable suggests that this protein has an additional function. Due to the proximity of the termination codon to the HPT (Fig. 4) and the presence of strains with both (G)8 and (G)9 sequences, this gene is a strong candidate for phase variation (52). The phase variation of this gene is currently being further investigated.

The insertion-deletion mutation in the *dca* mutants created for the competence experiments was generated using an *aphA-3* cassette designed by Ménard et al. (42) to be nonpolar. It is demonstrated here that the mutation in *dca* did not affect transcription of the 3′ gene, *murF*, and, given that the conserved *dcw* genes are essential, the effects on their transcription and translation should negatively impact the organism.

Due to the fact that the functions of the homologues of NMB0417 and NMB0419 are not known, the possibility still exists that this mutation is having an effect on the expression or function of those gene products, effecting the competence phenotype. Classical complementation studies, which could rule out this possibility, cannot be done due to the lack of genetic tools for complementation in the pathogenic *Neisseria* spp.

In summary, the *dcw* cluster of the pathogenic *Neisseria* spp. is highly homologous to those seen in other diverse organisms (Fig. 1). Similar to *E. coli*, there is a σ^{70} promoter located 5′ of *yabB*. It is not known if this one promoter drives transcription for the entire cluster, regardless of additional transcription from internal promoters, as P_{mra} is thought to do in *E. coli*. The three unique ORFs found within the highly conserved *dcw* cluster do not have features of recent horizontal acquisition (Fig. 2) but rather are integral parts of the genome. Unlike *B. subtilis*, which also has additional genes in its *dcw* cluster (12, 31), these are not homologues of other *dcw* genes. *dca*, one of these additional genes, encodes a putative inner membrane protein that has similarity to hypothetical inner membrane proteins present in several gram-negative bacteria that are located outside of their *dcw* clusters (Fig. 3). DNA sequencing of this ORF from several gonococcal and meningococcal strains revealed the presence of a homopolymeric tract of G's of variable length in this ORF, which suggests that this gene is phase variable (Fig. 4). We observed that while loss of the *dcw* cluster-associated ORF *dca* had no significant effect on peptidoglycan synthesis and growth rate, it had a profound effect on transformation, which could be localized to the 5′ region of the reading frame (Table 2). Thus, its loss rendered gonococci incapable of being transformed by chromosomal DNA, although meningococcal transformation was unchanged by similar mutagenesis of *dca*. We propose that Dca is a necessary component of the transformation machinery of gonococci, perhaps functioning to facilitate movement of DNA across the cytoplasmic membrane. The lack of necessity of Dca in the meningococci may suggest the presence of an alternate pathway in this species and that Dca may have additional, as yet undefined, functions.

ACKNOWLEDGMENTS

We thank L. Pucko for help with manuscript preparation and L. Melsen for his help with electron microscopy.

This work was supported by NIH grant AI-21150 (W.M.S.). W.M.S. is the recipient of a Senior Career Scientist Award from the VA Medical Research Service. N.J.S. was supported by a Wellcome Trust Research Fellowship in Medical Microbiology. The *N. gonorrhoeae* sequence was obtained from the University of Oklahoma, the Gonococcal Genome Sequencing Project (B. A. Roe, S. P. Lin, L. Song, X. Yuan, S. Clifton, T. Ducey, L. Lewis, and D. W. Dyer), which is supported by USPHS-NIH grant AI-38399. The *N. meningitidis* serogroup A strain Z2491 sequence and the *S. typhi* strain CT18 sequence were obtained from the Sanger Centre; these projects are supported by The Wellcome Trust. The *N. meningitidis* serogroup B strain MC58 sequence was obtained from The Institute for Genomic Research. The *N. meningitidis* serogroup C strain FAM18 sequence was obtained from the Sanger Centre; these projects are supported by Beowulf Genomics. The *A. actinomycetemcomitans* strain HK1651 sequence was obtained from the University of Oklahoma, the *Actinobacillus* Genome Sequencing Project (B. A. Roe, F. Z. Najar, S. Clifton, T. Ducey, L. Lewis, and D. W. Dyer), which is supported by a USPHS-NIH grant from the National Institute of Dental Research.

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