First evidence of the presence of genomic islands in *Escherichia coli* P4, a mammary pathogen frequently used to induce experimental mastitis

D. Dufour,* P. Germon,† E. Brusseau,* Y. Le Roux,* and A. Dary*¹

*URAFPA (Unité de Recherche sur l'Animal & Fonctionnalités des Produits Animaux), Equipe Protéolyse-Biofonctionnalité des Protéines et des Peptides, Nancy-Université, Vandœuvre-lès-Nancy, France

†INRA, UR1282 Infectiologie Animale et Santé Publique, F-37380 Nouzilly, France

ABSTRACT

Mastitis pathogens belonging to *Escherichia coli* species are often considered as environmental opportunistic pathogens that invade the udder and are rapidly killed by the immune system of cows. However, several studies have reported that some of these strains are able to persist in the udder for prolonged periods or to adhere and invade mammary epithelial cells, suggesting that they might possess some specific properties or genes that could be involved in their capacity to provoke mastitis. The aim of this work was to search for such specific genes in the *E. coli* strain P4, which was isolated from a case of severe mastitis and is often used to induce experimental mastitis. We established that this strain belongs to phylogenetic group A of the *E. coli* species, and that its core genome is very similar to that of the commensal nonpathogenic strain *E. coli* K-12 MG1655. Seventeen transfer RNA loci, known to be frequently associated with genomic islands, were screened and an altered structure was detected for 7 of them. The partial characterization of 5 of these loci (*asnT*, *leuX*, *pheV*, *serU*, and *thrW*) and the complete characterization of 1 (*argW*) revealed the presence of genomic islands that differ from those already described in pathogenic or nonpathogenic *E. coli* strains.

Key words: *Escherichia coli*, bovine mastitis, phylogeny, genomic island

INTRODUCTION

Bovine mastitis is an economically important disease in dairy cattle worldwide (Bradley, 2002). It is defined as an inflammation of the mammary gland caused by a bacterial infection. Depending on the occurrence of various clinical signs, mastitis is referred to as clinical (elevated rectal temperature, dullness, loss of appetite, diarrhea, or a swollen and painful udder) or subclinical

mastitis (Menzies et al., 2003). In both types of mastitis, milk production, as well as quality of milk, is affected. Production decreases considerably and milk quality declines strongly, particularly in regards to proteolysis of caseins—the major milk proteins (Michelutti et al., 1999). Among bacteria known to invade the udder and grow in this environment, *Escherichia coli* has been reported to be frequently responsible for clinical mastitis, and in rare cases, for fatal mastitis (Wenz et al., 2001; Burvenich et al., 2003; Shpigel et al., 2008).

Because of its ability to occupy different ecological niches, *E. coli* is considered to be a very versatile species (Bradley and Green, 2001; Touchon et al., 2009). Indeed, this species encompasses commensal and pathogenic isolates that can provoke different diseases: intestinal diseases ranging from mild diarrhea to severe colitis and dysentery, and extraintestinal diseases such as urinary tract infection or neonatal meningitis (Kaper et al., 2004). On the basis of their capacity to cause persistent or transient infection, mastitis pathogens have been divided into contagious or environmental pathogens, and, in this context, *E. coli* has been considered as an environmental pathogen (Nemeth et al., 1994). Several observations could account for such a classification. First, strains of *E. coli* isolated from mastitis cases belong to a large number of serotypes that are similar to those of fecal strains, whereas intestinal and extraintestinal pathogenic strains belong to a smaller number of serotypes (Burvenich et al., 2003). Second, no particular subset of genes involved in virulence and shared by all *E. coli* mastitis pathogens has been identified (Shpigel et al., 2008), virulence being defined here as the relative capacity to cause damage in a host (Casadevall and Pirofski, 2003). Numerous attempts have failed to identify, in various *E. coli* mastitis pathogens, genes encoding virulence factors known to be involved in the pathogenicity of intestinal or extraintestinal strains (Kaipainen et al., 2002; Lehtolainen et al., 2003; Ghanbarpour and Oswald, 2009). Finally, several studies have clearly showed that endotoxin or LPS is important during *E. coli* mastitis and that LPS constitutes a key molecule in the initiation of the inflam-

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¹Corresponding author: Annie.Dary@scbiol.uhp-nancy.fr

mation. Thus, intramammary administration of LPS partially mimics the inflammatory reaction observed during *E. coli* mastitis (Burvenich et al., 2003). Several authors have proposed that *E. coli* mastitis pathogens constitute opportunistic invaders of the mammary gland, capable of inducing an inflammatory reaction and that most of them are rapidly eliminated by the innate host immune system. It was also proposed that the severity of a clinical *E. coli* mastitis, which varies among field cases from mild to fatal, depends more on factors related to the cow, such as the periparturient period, the early stage of lactation, the parity, or some hormonal and metabolic alterations, than on the type of *E. coli* strain that has induced mastitis (Burvenich et al., 2003).

However, several reports have shown that some strains of *E. coli* isolated from mastitis cases possess additional properties that could be important or improve their virulence. For example, Döpfer et al. (2000, 2001) and Dogan et al. (2006) noted that some of these strains display adhesion and invasion properties when placed in the presence of bovine mammary epithelial cells (primary and MAC-T cell cultures). Moreover, Bradley and Green (2000, 2001) have shown that some strains of *E. coli* are able to access the bovine mammary gland during the nonlactating period and then persist until the onset of lactation and cause clinical mastitis. Thus, all of these results suggest that at least the *E. coli* mastitis pathogens studied in these works may possess the capacity to survive and adapt to the mammary gland, where many antibacterial entities are present such as polymorphonuclear cells, immunoglobulins, lactoferrin, complement, lysozyme, lactoperoxidase, or cytokines (Sordillo et al., 1997; Rainard and Riollot, 2006).

It is clearly established that the gene pool of a bacterial species consists of a set of genes shared by all strains of the species and that constitute the core genome and by genes encoding accessory functions, such as specific metabolic properties or virulence factors. These latter genes can differ from strain to strain and confer specificity to different strains. They are often clustered in so-called genomic islands (GEI) that have been acquired by horizontal gene transfer events (Dobrindt et al., 2004). Genomic islands have been sought and widely characterized in intestinal and extraintestinal pathogenic *E. coli* strains (Hacker and Kaper, 2000), and in commensal strains (Grozdanov et al., 2004; Hejnova et al., 2006), but not yet in strains isolated from mastitis cases even though these strains may contain genes that could contribute to the development of mastitis.

The aim of this work was to initiate the genetic characterization of the prototypical strain *E. coli* P4 originally isolated from a case of severe bovine mastitis (Bramley, 1976). This strain is often used to induce

experimental bovine mastitis and to study factors depending on the immune system of cows. It possesses the capacity to promote apoptosis and proliferation of bovine mammary cells (Long et al., 2001), and to adhere to or invade mammary gland epithelial cells in a murine model of mastitis (Gonen et al., 2007), suggesting that it could possess specific properties or genes that distinguish it from other commensal or pathogenic *E. coli* strains.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

Escherichia coli P4 (O32:H37, β -glucuronidase +, hemolysin –; Vangroenweghe et al., 2004) was provided by the Department of Comparative Physiology and Biometrics (C. Burvenich, Gent, Belgium). *Escherichia coli* O157:H7 EDL933 was used as a control, as well as *Escherichia coli* K-12, which was purchased from Institut Pasteur (Paris, France). *Escherichia coli* EPI100 (Epicentre, Madison, WI) was used as the recipient strain for the construction of a cosmid library of *E. coli* P4 genome, and the cosmid pWEB:Tnc (Epicentre) was used as vector.

The *E. coli* strains were grown in Luria-Bertani medium (Sambrook et al., 1989) with or without 1.5% (wt/vol) Bacto Agar (Difco Laboratories, Detroit, MI) at 37°C. Recombinant strains were grown in Luria-Bertani medium supplemented with ampicillin (100 μ g/mL; Euromedex, Souffelweyersheim, France). All strains were stored at –70°C in Luria-Bertani medium supplemented with glycerol (20% wt/vol).

DNA Isolation, Electrophoresis, and Southern Hybridization

Genomic DNA of *E. coli* P4, *E. coli* K-12 MG1655, and *E. coli* O157:H7 EDL933 was isolated as described previously (Leblond et al., 1996). For the construction of the cosmid library of *E. coli* P4 genome, genomic DNA of *E. coli* P4 was isolated as described previously (Sambrook et al., 1989). Recombinant cosmids were extracted by the alkaline lysis method (Sambrook et al., 1989). Purification of DNA fragments and extraction from agarose gel slices were performed with NucleoSpin Extract II system (Macherey-Nagel, Hoerdtt, France). The DNA fragments were separated on agarose gels according to Sambrook et al. (1989). Both 1-kb and 100-bp DNA ladders (Bio-Rad, Hercules, CA) were used as molecular weight markers. Southern blotting, prehybridization, and hybridization conditions were used as described previously by Demuyter et al. (1988). Labeling of probes and detection of specific hybrids

were performed using the DIG DNA labeling and detection kit, under the conditions recommended by the supplier (Roche Molecular Biochemicals, Mannheim, Germany).

Cosmid Library Construction and Screening

Genomic DNA of *E. coli* P4 was partially digested with the restriction endonuclease *Sau3AI* (New England Biolabs, Beverly, MA) and ligated to the *Bam*HI (New England Biolabs) digested cosmid vector pWEB:Tnc that had been pretreated with alkaline phosphatase (New England Biolabs). The recombinant cosmids were packed in phage heads with the Gigapack III XL system (Stratagene, Heidelberg, Germany), according to the instructions of the manufacturer, and used to infect *E. coli* EPI100. Recombinant cosmids containing the 6 identified altered transfer (t)RNA (*argW*, *aspV*, *leuX*, *pheV*, *serU*, and *thrW*) loci identified were first screened by PCR using 6 pairs of primers designed according to the nucleotide sequences of the region located downstream from these 6 tRNA loci and determined using an arbitrary PCR followed by a sequencing of the products. The PCR products obtained using these pairs of primers, and the *E. coli* P4 genomic DNA as template, were also used as probes in Southern hybridization experiments to confirm the identified recombinant cosmids. These probes were labeled and hybridized against *Eco*RI (New England Biolabs) restriction digest of the identified recombinant cosmids.

PCR Primers

All PCR primers used in this study (Table 1) were purchased from Eurogentec Society (Liège, Belgium). Primers enabling the partial amplification of the genes *chuA*, *yjaA*, and *tspE4C2* were used to assign the strain *E. coli* P4 to 1 of the 4 phylogenetic groups of the *E. coli* species (Clermont et al., 2000) as follows: *chuA* positive and *yjaA* positive = group B2; *chuA* positive and *yjaA* negative = group D; *chuA* negative and *tspE4C2* positive = group B1; and *chuA* negative and *tspE4C2* negative = group A.

Primers used for multilocus sequence typing (MLST) enabled the partial amplification of different house-keeping genes in the genome of *E. coli* K-12 MG1655: *adk* (adenylate kinase), *gcl* (glyoxylate carboligase), *gdh* (glucose-6-phosphate dehydrogenase), *mdh* (malate dehydrogenase), *metA* (homoserine transsuccinylase), *ppk* (polyphosphate kinase), *aspC* (aspartate aminotransferase), *clpX* (ATPase and specificity subunit of ClpX-ClpP ATP-dependent serine protease), *fadD* (acyl-CoA synthetase), *icdA* (isocitrate dehydrogenase), *lysP* (lysine transporter), *aroE* (shikimate dehydroge-

nase), *cstA* (carbon starvation protein), *cyaA* (adenylate cyclase), *dnaG* (DNA primase), *grpE* (heat shock protein), *mtlD* (mannitol 1-phosphate dehydrogenase), *mutS* (methyl-directed mismatch repair protein), *rpoS* (sigma factor 38), and *pgi* (phosphoglucose isomerase) (Reid et al., 2002; Adiri et al., 2003). Additional primers were designed for the partial amplification of genes encoding the serine endoprotease Do (*degQ*) and the following putative proteases of unknown function in the genome of *E. coli* K-12 MG1655: *ptrB* (proteinase II), *ycaL* (putative protease), *yhjS* (putative protease), *yaeL* (zinc metalloproteinase), *sms* (probable ATP-dependent protease), *yhjJ* (predicted zinc-dependent peptidase), *elaD* (predicted enzyme), and *pmbA* (predicted peptidase required for the maturation and secretion of the antibiotic peptide MccB17). The presence of the serum resistance factor *traT* was evaluated using primers already described by Kaipainen et al. (2002).

The primers of Dezfulian et al. (2003) were used to search potential GEI at the *leuX*, *metV*, *selC*, and *thrW* tRNA loci, whereas the primers of Germon et al. (2007) were used to search for GEI at the *asnT*, *asnUVW*, *aspV*, *glyU*, *pheU*, *pheV*, *serU*, *serX*, and *serW* tRNA loci. For the *argU* and *argW* tRNA loci, GEI were searched using the primers designed from the *E. coli* K-12 MG1655 genome.

To amplify the first nucleotides of sequences located downstream of the interesting tRNA loci, arbitrary PCR consisting of 2 steps were used. The first corresponded to a PCR using genomic DNA of the strain as template, a first forward primer targeting the tRNA gene (*argW*1ter, *asnT*-F, *aspV*-F, *leuX*1, *pheV*3bis, *serU*3, and *thrW*1) and the degenerated reverse primers ARB1, ARB3, or ARB4. The second step corresponded to a PCR using an aliquot (2 µL) of the products obtained during the first PCR as template, a second forward primer targeting a sequence of the same tRNA gene but located downstream from the region targeted with the first forward primer (*argW*-F, *asnT*3bis, *aspV*3, *leuX*3, *pheV*-F, *serU*-F, and *thrW*3), and the reverse primer ARB2.

Nine pairs of primers were designed to amplify the entire prophage-like element SpLE5 localized downstream from the *leuX* tRNA locus in *E. coli* O157:H7 Sakai genome (GenBank accession number: BA000007.2).

To screen recombinant cosmids containing the 6 identified altered tRNA loci identified (*argW*, *asnT*, *leuX*, *pheV*, *serU*, and *thrW*) 6 pairs of primers were designed according to the nucleotide sequences of the region located downstream from the interesting loci and determined by arbitrary PCR.

Finally, the entire genomic island located downstream from the *argW* tRNA locus was amplified with the forward primer *argW*3bis, designed according to the first

Table 1. Primers for PCR designed during this study

PCR type and target gene	Primer ¹	Primer sequence (5' to 3')	Annealing temperature (°C)	PCR product size (bp)
Multilocus sequence typing simplex PCR				
degQ	degQ-F	ATCAACCTGCACAACCCCTTC	58	650
	degQ-R	CAACACTTCGCTGACAAACG		
yaeL	yaeL-F	GTCCGGTTGCAAACCTTCATT	58	657
	yaeL-R	GCCCATACTGGCGTACAACCTT		
sms	sms-F	CTGCCGACTGACAATCTCAA	58	652
	sms-R	CCACGTTTCAAAAACACATCC		
yhjJ	yhjJ-F	CGGCAGTCAAATCCAAGACC	56	647
	yhjJ-R	GGTAATTGTCTCTTATGA		
elaD	elaD-F	TTCAGAAGATCGCCGCTAAT	56	676
	elaD-R	TTGCCGGATGCTCTATTTTTT		
pmbA	pmbA-F	ATCACCAATACCGAAGGTGG	60	646
	pmbA-R	CCCGGTGCCCATCTCTTTGAG		
ptrB	ptrB-F	CCAGTTTGCGCCAAATTAAC	58	648
	ptrB-R	CGATAACGCCGTGGAATAAT		
ycaL	ycaL-F	GCGACCTCTGCAGCATTACT	60	644
	ycaL-R	TCCAGGCTAGCCAGTGTTC		
yhjS	yhjS-F	GAAGAGGCGGAGATCCAAC	58	648
	yhjS-R	AAACAGCACCAGCCGATTAC		
Transfer (t)RNA screening simplex PCR				
argU	argU-F	CGCCCTTAGCTCAGTTTGA	55	483
	argU-R	TGTTGCGTGATCAAATAGGC		
argW	argW-F	TAGTTAAATGGATATAACGAGC	55	600
	argW-R	TTTTCGCCTTTACAGGATCG		
<i>argW</i> locus long-range PCR				
argW	argW3bis	TTGGGATCTATCCCCCTTGAC	62	
dsdA	dsdA-R	GCTGCTTTACGTCCCTTCCTC		
Screening of recombinant cosmid PCR				
argW	argW3	TGCAAAGCAGCTAAGCCACG	55	250
	argW3bis	TTGGGATCTATCCCCCTTGAC		
asnT	asnT5bis	ATGTCACTGGTTTCGAGTCC	55	191
	asnT6bis	GGGGTCACTCCAGAATCG		
leuX	leuX1	GCAGGACAGTGTGAGATTAC	55	267
	leuX19	CCGTCGCCAGCCAGAATAC		
pheV	pheV5	GATGATAGATTGTGCAGTCTGC	55	500
	pheV6	ACGAGACGAGGCGAATCAG		
serU	serU4	GTCGCGTACAGGCTAACC	55	592
	serU5	CTGTGCTATACCAGCACC		
thrW	thrW11	ACACATCATTAGTACCTTCC	55	256
	thrW12	AAAGCGGCGCTGAGACGG		
Screening of SpLE5 PCR/target position in <i>E. coli</i> O157:H7 Sakai genome				
534725	leuX1	GCAGGACAGTGTGAGATTAC	58	5,001
5352052	leuX2	AAGCTGCCGTTTTTACCTCA		
5352173	LeuX3	CAACCCTAAGGCCATCTGAG	60	5,001
5356975	LeuX4	AAGCCATTCAAGCGTCAAAGTAA		
5357133	LeuX5	AAGGCAGGTATGGCACGTTA	58	4,803
5361933	LeuX6	CTGGGCTGATGATGGTTTTT		
5362177	LeuX7	TTGCTTGCTCAAAAAGCATGTA	60	4,790
5366967	LeuX8	AGCTGTCGGGGTTCCCTTAAT		
5367145	LeuX9	AACTGTCTGCGCTTTTGAAT	58	4,805
5371948	LeuX10	TTTATTGCTCCCTGACGAT		
5372276	LeuX11	CTCGATTGCAGTTGCGATAA	58	5,001
5377073	LeuX12	CTTCTGATCGTGTGCTTCA		
5377199	LeuX13	CTCTCTACGGCGGTCTTCAC	60	5,001
5381993	LeuX14	GCGTTCATCGGGTAGATGAT		
5382112	LeuX15	ATGTCGGCAGAAAAGGTCATT	60	5,001
5386910	LeuX16	TCTGCGACGCTATTTTTGTGGA		
5387111	LeuX17	AACGCAACAGTTTTGCACTG	58	5,001
5391906	LeuX18	TGGTTGAAGCCTTTTCGTAGG		
Arbitrary PCR/target gene or factor				
	ARB1	GGCCACGGCGTCTGACTAGTACNNNNNNNNNNNGACTG		
	ARB2	GGCCACGCGTCTGACTAGTAC		
	ARB3	GGCCACGCGTCTGACTAGTACNNNNNNNNNNNGAATG		
	ARB4	GGCCACGCGTCTGACTAGTACNNNNNNNNNNNGATTCCG		

Continued

Table 1 (Continued). Primers for PCR designed during this study

PCR type and target gene	Primer ¹	Primer sequence (5' to 3')	Annealing temperature (°C)	PCR product size (bp)
<i>argW</i>	argW1ter	CTCCATTAGCGGGTATACTCATGCC		
	argW-F	TAGTTAAATGGATATAACGAGC		
<i>asnT</i>	asnT-F	TAAGTAATATGCGCCCGTTC		
	asnT3bis	TAGTTCAGTCGGTAGAACGG		
<i>aspV</i>	aspV-F	GGCGGAAGCTGCCTCTGGCG		
	aspV3	TTCCCAACGGAACACAACGC		
<i>leuX</i>	leuX1	GTGGCGTGCACAGGTATA		
	leuX3	CGTTTTCCGCATACCTCTTC		
<i>pheV</i>	pheV3bis	GATGATAGATTGTGCAGTCTGC		
	pheV-F	ACGAGACGAGGCGAATCAG		
<i>serU</i>	serU3	GAACAAAATTGCACGGACAG		
	serU-F	TCCTGGCATCATGGCAACC		
<i>thrW</i>	thrW1	AAGGCCATTGACGCATCGC		
	thrW3	TGAACATGTCCTTTCAGGGC		

¹F = forward, R = reverse.

nucleotide sequence of the region located downstream from the *argW* locus, which was amplified by arbitrary PCR and sequenced, and with the reverse primer dsdA-R, designed from the complete genome sequence of *E. coli* K-12 MG1655.

PCR Amplification

A My Cycler thermocycler (BioRad, Bristol, UK) was used for the different amplification procedures, which are presented in Table 2. The PCR reactions were performed in a volume of 50 µL with about 200

ng of genomic DNA. Either 1 or 1.25 U of Taq DNA Polymerase (BioRad) was routinely used, except for the arbitrary PCR, in which 5 U was used. One unit of Accu Taq DNA polymerase (Sigma, Dorset, UK) was used for the screening of the *asnUVW*, *pheV*, *aspV*, *glyU*, and *serX* tRNA loci and of the prophage-like element SpLE5 PCR, whereas 2.5 U was used for the amplification of the *argW* tRNA locus. Two hundred micromoles of each dNTP (BioRad) was used for each PCR, except for the *traT* gene PCR, in which 100 µM of each dNTP was used, and for the *argW* tRNA locus, in which 500 µM of each dNTP were used. One micro-

Table 2. Polymerase chain reaction amplification procedures used in this study

Procedure	Initial denaturation step	Amplification cycles ¹	Number of cycles	Final elongation step
Phylogenetic group, multilocus sequence typing, <i>traT</i> gene, and screening of recombinant plasmid PCR	94°C for 5 min	94°C for 30 s T-30 s 72°C for 30 s	30	72°C for 7 min
Screening transfer (t)RNA of <i>asnT</i> , <i>argU</i> , <i>argW</i> , <i>leuX</i> , <i>metV</i> , <i>pheU</i> , <i>pheV</i> , <i>selC</i> , <i>serU</i> , <i>serW</i> , <i>thrW</i> loci PCR	94°C for 5 min	95°C for 1 min T-1 min 72°C for 1 min	30	72°C for 7 min
Screening tRNA of <i>asnUVW</i> , <i>aspV</i> , <i>glyU</i> , <i>serX</i> loci and SpLE5 PCR	94°C for 5 min	95°C for 1 min T-1 min 72°C for 5 min	30	72°C for 7 min
Internal transcribed spacer 16S-23S PCR	95°C for 3 min	95°C for 1 min T-50 s 72°C for 2 min	35	72°C for 10 min
<i>argW</i> tRNA locus long-range PCR	94°C for 1 min	98°C for 10 s T-30 s 72°C for 20 min	30	72°C for 10 min
First step of arbitrary PCR	95°C for 5 min	95°C for 30 s 30°C for 30 s 72°C for 2 min	6	None
		95°C for 30 s 45°C for 30 s 72°C for 2 min	30	
Second step of arbitrary PCR	95°C for 5 min	95°C for 30 s 52°C for 30 s 72°C for 2 min	30	None

¹T = annealing temperature specified for each PCR type or target gene in Table 1.

mole of each primer was used for each PCR, except for the *argW* tRNA locus, *traT* gene, and arbitrary PCR, in which 0.4, 0.3, and 0.8 μ M were used, respectively.

DNA Sequence Analysis, Sequence Annotation, and Nucleotide Sequence Accession Numbers

Sequencing was carried out by the Genome Express Society (Grenoble, France) according to the method of Sanger. Homology searches of homology were carried out using the BLAST programs of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). Putative conserved domain sequences (CDS) were identified using the CDS Finder program of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>), and the Glimmer program of the Nano + Bio Center (<http://www.nbz.uni-kl.de/>). Conserved domains of putative CDS were searched by using the Conserved Domain Database (CDD) program of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Ribosome binding sites (RBS) and rho-independent terminator were searched by using the RBS Finder and Trans Term programs of the Nano + Bio Center (<http://www.nbz.uni-kl.de/>).

Escherichia coli P4-specific sequences of the *cyaA*, *grpE*, *mtlD*, *elaD*, *pmbA*, and *sms* genes, and of the *argW*, *asnT*, *leuX*, *pheV* (2 sequences), *serU*, and *thrW* tRNA loci determined in this study were submitted to the EMBL nucleotide sequence database, and are available under accession numbers EU876896, EU876897, EU876898, EU876899, EU876900, EU876901, and EU876907, EU876902, EU876904, EU876905-EU876906, EU876908, and EU876903, respectively.

RESULTS

Phylogenetic Analyses of *E. coli* P4

We began this study by determining to which of the 4 main phylogenetic groups (A, B1, B2, and D) of the *E. coli* (Selander et al., 1987; Herzer et al., 1990) the strain P4 belonged. To this end, we used the PCR assay described by Clermont et al. (2000). The strain *E. coli* K-12 MG1655 was used as control. Only the *yjaA* gene was detected in *E. coli* P4 (and confirmed by sequencing), as in the control *E. coli* K-12 MG1655, suggesting that *E. coli* P4 belongs to the phylogenetic group A.

This result was confirmed by performing a MLST analysis, based on the sequence comparison of several genes belonging to the core genome of the *E. coli* species. An internal fragment of 29 genes, most of which had been already used in several MLST studies, was thus amplified from *E. coli* P4 and sequenced. Among the 29 core genes, only 6 did not share 100% identity with

the corresponding genes of *E. coli* K-12 MG1655. The *cyaA*, *grpE*, *mtlD*, *elaD*, and *pmbA* sequences shared 99.8, 99.75, 99.81, 99.75, and 98.72% identity with those of *E. coli* K-12 MG1655, respectively. In each case, they remained closer to the same genes in the *E. coli* K-12 MG1655 genome. Conversely, the *sms* sequence shared 98.25% identity with that of *E. coli* O157:H7 Sakai. By comparing these results with those of the EcMLST database (Reid et al., 2002), we confirmed that *E. coli* P4 actually belongs to the phylogenetic group A and is phylogenetically closer to the *E. coli* K-12 MG1655 strain, a commensal and nonpathogenic strain of the phylogenetic group A.

Detection of GEI in *E. coli* P4 Genome by tRNA Screening

We then investigated whether *E. coli* P4 could contain potential virulence genes. We searched by PCR for several virulence factors known in other *E. coli* pathotypes to confer cellular adhesion, invasion, or proteolytic properties, but no amplicon was obtained (data not shown). Finally, only the *traT* gene, encoding a serum resistance factor, was detected by PCR in the genome of *E. coli* P4 and confirmed by sequencing.

Genes conferring specific properties to particular *E. coli* strains are frequently located on GEI, themselves often being located downstream of tRNA genes (Hou, 1999). To identify new genes in the *E. coli* P4 strain that could explain its specific properties, we thus looked for potential GEI downstream of tRNA genes using a PCR-based tRNA screening to detect sequence context alterations around the tRNA genes. Because the above phylogenetic analyses indicated that *E. coli* P4 was closely related to *E. coli* K-12 MG1655, this strain was used as a control. Size differences between the PCR products obtained from the 2 genomes or the absence of the expected amplicon from the genome of *E. coli* P4 indicated a genomic variation and possibly a chromosomal integration of foreign DNA at the 3' end of the tRNA gene tested. We screened 17 (*argU*, *argW*, *asnT*, *asnUVW*, *aspV*, *glyU*, *leuX*, *metV*, *pheU*, *pheV*, *selC*, *serU*, *serW*, *serX*, and *thrW*) out of 36 likely acceptor tRNA genes because they had been previously shown to be the loci that are the most frequently associated with GEI (Germon et al., 2007). In strain P4, sequence context variations were detected in 7 of them (*argW*, *asnT*, *aspV*, *leuX*, *pheV*, *serU*, and *thrW*). For *argW*, *asnT*, *aspV*, *leuX*, *serU*, and *thrW* loci, no amplicon was obtained, whereas one amplicon of unexpected size (2,208 bp vs. 623 bp for K-12) was obtained for the *pheV* locus. Therefore, these results suggested the presence of genomic variations at the 3' end of these 7 tRNA loci in the *E. coli* P4 genome.

Sequence Analysis of the Region Located Downstream from the *argW*, *asnT*, *leuX*, *pheV*, *serU*, and *thrW* tRNA Loci

The regions located immediately downstream from 6 (*argW*, *asnT*, *leuX*, *pheV*, *serU*, and *thrW*) out of the 7 altered tRNA loci were amplified from the *E. coli* P4 genome by arbitrary PCR. Fragments of 747 bp (*ArgW*-P4), 202 bp (*AsnT*-P4), 1,335 bp (*LeuX*-P4), 1,677 bp (*PheV*-P4), 755 bp (*SerU*-P4), and 696 bp (*ThrW*-P4) were obtained for the *argW*, *asnT*, *leuX*, *pheV*, *serU*, and *thrW* loci, respectively, and were sequenced (Table 3). Their G+C content ranged from 40.1 to 47.56%. In each case, the sequences shared similarities with a sequence located at the same locus in another *E. coli* strain. An open reading frame (ORF) encoding a putative integrase was observed in the *leuX*-P4 and *pheV*-P4 sequences (Table 3). It appeared to be functional in the case of *pheV*-P4 and truncated in the case of *leuX*-P4. Because the integrase detected in this latter case shared similarities with an integrase of the prophage-like element SpLE5 of *E. coli* O157:H7 Sakai, 9 pairs of primers specific to this prophage were designed and used in PCR reactions to determine whether such an element was present in *E. coli* P4. Genomic DNA of *E. coli* O157:H7 EDL933 and *E. coli* K-12 MG1655 were used as positive and negative controls, respectively. No amplicon with the expected size was obtained for strain P4: PCR products were either smaller than expected or completely absent. The PCR products obtained with the primers *leuX*1/X2, *leuX*3/X4, *leuX*5/X6, *leuX*13/X14, *leuX*15/X16, and *leuX*17/X18 were sequenced. They displayed 70 to 98% identity with the corresponding regions of the SpLE5 element. Thus, it appeared that an altered structure of the prophage-like element SpLE5 is present in the *E. coli* P4 genome. Finally, the 2,208-bp PCR product obtained during the *pheV*-tRNA screening (see above) was also sequenced. This sequence had a G+C content of 49.60% and displayed identities (>80%) with different genomic fragments of the LEE-PAI of the bovine shiga toxin-producing *E. coli* RW1473 strain (GenBank accession number: AJ303141). Its last 333 nucleotides also displayed 100% identity with the first 333 nucleotides of the *yghD* gene of *E. coli* K-12 MG1655 genome, which is the first conserved gene found in the *E. coli* genomes downstream from the *pheV* tRNA locus. From the positions 1,705 to 1,727, a 23-bp sequence was identified that was 100% identical to the last 23 nucleotides of *pheV* gene. This 23-bp sequence has been previously identified as the target of recombination events at the *pheU* and *pheV* loci in enteropathogenic and enterohemorrhagic *E. coli* (Rumer et al., 2003). Consequently, this 2,208-bp region seems to represent the 3' end of a

GEI located downstream from the *pheV* locus in the *E. coli* P4 genome. Computer analysis revealed the presence of 3 ORF (ORF1-*pheV*, 2-*pheV*, and 3-*pheV*, Table 4). They encoded hypothetical proteins similar to proteins with unknown function of the commensal strain *E. coli* Nissle 1917, the enteropathogen strain *E. coli* B7A, and the bovine enterohemorrhagic strain *E. coli* RW1374, respectively. Thus, the region located downstream of the *pheV*-tRNA locus in the *E. coli* P4 genome seemed to have a composite structure.

To conclude, analyses of the regions located immediately downstream of the *argW*, *asnT*, *leuX*, *pheV*, *serU*, and *thrW* loci of *E. coli* P4 revealed, in each case, presence of sequences different from those present at the corresponding loci in the *E. coli* K-12 MG1655 genome. In the case of the *pheV* locus, the analysis of this sequence strongly suggested the presence of a GEI in *E. coli* P4, whereas no foreign DNA was inserted at this locus in *E. coli* K-12 MG1655 genome. For the *leuX* locus, an altered structure of *E. coli* O157:H7 Sakai SpLE5 seemed to be present in *E. coli* P4 genome, whereas another prophage-like element (KpLE2) with different genomic content is identified at this locus in *E. coli* K-12 MG1655 genome.

Partial Sequence Analysis of the GEI Located Downstream from the *serU* tRNA Locus in *E. coli* P4 Genome

To characterize the regions located downstream from the identified altered tRNA loci in *E. coli* P4, a genomic library was constructed using pWEB:Tnc as vector. Recombinant cosmids containing the regions of interest were selected by PCR and reconfirmed by hybridization using appropriate PCR products specific to the targeted regions as probes. One recombinant cosmid containing the *serU* locus was identified and partially sequenced. A nucleotide sequence of a 13,202-bp region downstream from the *serU* locus was determined. This sequence presented a G+C content of 42.5%. Seven ORF (Figure 1) were detected, 4 of which (ORF3-*serU*, 5-*serU*, 6-*serU*, and 7-*serU*) encode putative proteins with DNA mobility function, such as transposases and integrases. The ORF1-*serU* encodes a protein that is related to glycosylation function of the O-antigen part of LPS, the ORF4-*serU* encodes a protein of unknown function, and the protein encoded by the ORF2-*serU* has no significant similarity with any protein of the GenBank database. Proteins similar to those encoded by these 7 ORF seem to have different origin such as bacteriophage DNA (ORF1-*serU*, and 7-*serU*), bacterial chromosome DNA (ORF3-*serU*, and 6-*serU*), and bacterial plasmid (ORF4-*serU*, and 5-*serU*, which both are components of the *Th1000* of the F plasmid). Thus,

Table 3. Characteristics of the sequences ArgW-P4, AsnT-P4, LeuX-P4, PheV-P4, SerU-P4, and ThrW-P4

Transfer (t)RNA locus	Sequence	G+C content (%)	Ribosome binding site	Open reading frame position	Predicted size (AA)	Characteristics of homologous sequence			
						Identity/ similarity (%)	Homologous fragment or putative function	Species and strain	Accession number
argW	argW-P4	43.11	—	—	—	94	627-bp fragment	<i>Escherichia coli</i> APEC BEN1588	DQ023206
asnT	asnT-P4	40.1	—	—	—	96	195-bp fragment	<i>E. coli</i> C ATCC8739	CP000946
leuX	LeuX-P4	46.59	AGGCGG and AGGGT	91–1065	324	69/76	Integrase	<i>E. coli</i> O157:H7 Sakai	BAB38665
pheV	pheV-P4	46.57	—	198–1460	420	90/95	P4-type integrase	<i>Shigella flexneri</i> 2a strain 301	AAN44445
serU	SerU-P4	44.11	—	—	—	79	716-bp fragment	<i>E. coli</i> HS	CP000802
thrW	ThrW-P4	47.56	—	—	—	98	696-bp fragment	<i>S. flexneri</i> 2a strain 301 and <i>E. coli</i> O157:H7 Sakai ¹	—

¹In *S. flexneri* 2a 301 and *E. coli* O157:H7 Sakai, the corresponding sequences contain the last moiety and the last two-thirds of an open reading frame encoding a putative integrase.

Table 4. Open reading frames (ORF) detected within the 1,875 bp located downstream from the *pheV*-tRNA locus

ORF	Position	Ribosome binding site	G+C content (%)	Predicted size (AA)	Accession number	Conserved domain	Characteristics of homologous protein			
							Function and accession number	Species (strain)	Length (AA)	Identity/ similarity (%)
ORF1- <i>pheV</i>	370–816	—	53.2	148	ACG50150	—	Hypothetical protein (CAE55810)	<i>Escherichia . coli</i> (Nissle 1917)	143	91/94
ORF2- <i>pheV</i>	683–925	—	49.6	80	ACG50151	—	Hypothetical protein (ZP_0076238.1)	<i>E. coli</i> (B7A)	80	98/100
ORF3- <i>pheV</i>	1,022–1,594	AGGAA, 1,009–1,013	45.8	190	ACG50152	—	Hypothetical protein (CAI43851)	<i>E. coli</i> (RW1374)	190	98/98

Figure 1. (A) Partial genetic map of the *serU* genomic region of *Escherichia coli* P4; (B) characteristics of the open reading frames (ORF) detected within the *E. coli* P4 *serU* genomic island partially characterized. RBS = ribosome binding site, sequence and position; CD = conserved domain.

this partial sequence suggests that the *serU* region of *E. coli* P4 genome presented a mosaic structure.

Sequence Analysis of the GEI Located Downstream from the *argW* tRNA Locus in *E. coli* P4 Genome

General Features. As the ArgW-P4 sequence presented no homology with any of the sequenced *E. coli* genomes, we decided to sequence the entire variable genomic region located downstream from the *argW* locus. A forward primer specific to the ArgW-P4 sequence

and a reverse primer specific to the sequence of the *dsdA* gene (first conserved gene found in the *E. coli* genome downstream from the *argW* tRNA locus) were designed and used to carry out long-range PCR. An amplicon of about 12 kb was obtained and sequenced. After removing the nucleotide fragments corresponding to the *argW* and *dsdA* genes, a genomic region of 11,253 bp was defined. It displayed an averaged G+C content of 40.92% and varied across this region from 31.2 to 48.73% (Figure 2). From the positions 8,628 to 8,650, a 23-bp sequence was identified that was identical to the

A.

B.

ORF	Position	RBS	G+C content (%)	Predicted size (aa)	Accession number	CD	Characteristic of homologous protein			
							Function (Accession number)	Species (strain)	Length (aa)	%Identity/%similarity
ORF1 - <i>argW</i>	165-1355	AGGGG (127-131)	43.07	396	ACG50154	P4 integrase (partial)	Integrase (YP_153333)	<i>Salmonella enterica</i> (ATCC9150)	419	68/82
ORF2 - <i>argW</i>	1512-1703	GAGG (1503-1506)	46.88	63	ACG50155	Transcriptional regulator (entire)	Predicted transcriptional regulator (ZP_00834742)	<i>Yersinia intermedia</i> (ATCC29909)	65	73/91
ORF3 - <i>argW</i>	2671-3723		43.59	350	ACG50156	/	Putative replication protein (NP_311263)	<i>E. coli</i> (O157:H7 Sakaï)	231	97/99
ORF4 - <i>argW</i>	6910-4430	AAGAG (6921-6917)	31.20	826	ACG50157	Sir2 (entire)	Hypothetical protein (YP_487138)	<i>Rhodopseudomonas palustris</i> (HaA2)	792	23/41
ORF5 - <i>argW</i>	7564-7388		43.50	58	ACG50158	/	Hypothetical protein (NP_311266)	<i>E. coli</i> (O157:H7 Sakaï)	58	77/84
ORF6 - <i>argW</i>	7867-8493		47.69	208	ACG50159	Recombinase/Resolvase (entire)	Putative resolvase (NP_311267)	<i>E. coli</i> (O157:H7 Sakaï)	208	98/99
ORF7 - <i>argW</i>	9681-8746	ATGAG (9692-9688)	48.08	311	ACG50160	Transcriptional regulator (entire)	DsdC transcriptional regulator (AAC75424)	<i>E. coli</i> (K12 MG1655)	311	100/100
ORF8 - <i>argW</i>	9899-11236	AGGGG (9884-9888)	48.73	445	ACG50161	Transporter (entire)	DsdX predicted transporter (AAC75425)	<i>E. coli</i> (K12 MG1655)	445	100/100

Figure 2. (A) Genetic map of the *argW* genomic region of *Escherichia coli* P4; (B) characteristics of the open reading frames (ORF) detected within the *E. coli* P4 *argW* genomic island. RBS = ribosome binding site, sequence and position; CD = conserved domain.

last 23 nucleotides of *argW* gene. Thus, this genomic region exhibited several features typical of GEI: a chromosomal integration site located downstream from a tRNA locus, direct repeat of the 3' end of this tRNA locus, large size, and atypical G+C content.

ORF Content. Eight ORF were detected in this 11,253 nucleotides sequence (Figure 2). The ORF1-*argW* and ORF6-*argW* encoded proteins with DNA mobility function; the ORF2-*argW*, ORF3-*argW*, and ORF7-*argW* encoded proteins with replication or transcription functions; the ORF8-*argW* encoded a protein with transport function; the ORF5-*argW* encoded a protein of unknown function; and the ORF4-*argW* encoded a protein with deacetylation function. The intergenic regions presented no similarity to any nucleotide sequence in the databases.

The deduced AA sequence of the putative protein encoded by the ORF1-*argW* presented 68% identity and 82% similarity with the AA sequence of an integrase of the *Salmonella enterica* ATCC 9150 strain. The ORF1-*argW* conserved only the N-terminal domain of integrase; that is, the DNA binding domain, and lost the catalytic C-terminal domain. The C-terminal domain of the ORF1-*argW* presented no significant degree of similarity with any protein sequence in the GenBank database. Consequently, the protein encoded by the ORF1-*argW* could be considered a chimeric protein, resulting from the association of a N-terminal domain of a tyrosine recombinase with a fragment of unknown origin. The last 2 ORF detected (ORF7-*argW* and ORF8-*argW*) showed 100% identity with the 2 ORF located upstream from the *dsdA* gene in the *E. coli* K-12 MG1655 genome; that is, *dsdC* and *dsdX* genes, respectively.

Sequence Analysis of ORF4-*argW*. The ORF4-*argW* was the largest ORF detected in the *argW* GEI of *E. coli* P4. It consisted of 2,481 nucleotides and presented a G+C content of 31.20%, which was significantly lower than the average in *E. coli*. A predicted Shine-Dalgarno site (AAGAG) was identified 11 bp upstream from the proposed start codon. A possible rho-independent transcriptional termination signal was also identified 14 bp downstream from the proposed termination codon. This ORF4-*argW* encoded a putative protein of 826 AA, with a deduced molecular mass of 95.18 kDa. From the position 27 to 223 of the AA sequence of this putative protein, a conserved domain of Silent Information Regulator 2 (Sir2) was detected. The Sir2 domain is characteristic of enzymes that catalyze NAD⁺-dependent protein/histone deacetylation. Such proteins have been identified in organisms ranging from bacteria to humans. They contain a series of consensus motifs grouped in a region of about 200 AA. The position of this region is highly variable in the

total sequence of the Sir2 proteins, and AA sequences around this region can also be highly variable. Prokaryotic Sir2 proteins lack certain consensus motifs, as was the case for the protein encoded by the ORF4-*argW* of *E. coli* P4, in which the consensus motifs GAGISXXX-GIPXXR, TQNID, GTS, and (I/V)N were effectively identified with an altered composition (GAGFSRKT-KNITNGEPPLA, TTNYD, GYS, and IV, respectively; Figure 3), and the consensus motifs PXXXH, HG, 2 sets of CXXC, and FGE were not identified. BlastP analyses showed that the N-terminal region of the putative protein encoded by ORF4-*argW* was similar to that of Sir2 proteins. Its C-terminal region presented no significant degree of similarity with any protein sequence in the GenBank database.

DISCUSSION

Mastitis is a worldwide problem in dairy cattle and leads to multibillion-dollar economic losses because of treatment, culling, death of infected cows, and important losses of milk production (Sordillo and Streicher, 2002). Among bacteria responsible for this disease, *E. coli* is an important cause of clinical mastitis and yet the mastitis pathogens of this species remain poorly characterized compared with other pathogenic strains of *E. coli*. To our knowledge, no particular subset of genes shared by all strains of *E. coli* isolated from mastitis cases, which could permit better understanding their pathogenic properties, has been identified in epidemiological studies (Shpigel et al., 2008). It has been proposed that their virulence rather relies on microbe-associated molecular patterns such as LPS, which are present in all of these strains and that are detected by the host innate immune system (Shpigel et al., 2008).

We focused our attention on the *E. coli* strain P4 for several reasons. First, this strain has been isolated from a case of severe mastitis and is widely used to induce experimental mastitis. Second, *E. coli* P4 is able to grow in a high antibacterial environment such as milk obtained from mastitis induced with LPS (Dufour et al., 2009). Third, it has been reported that this strain displays adhesion or invasion properties on mammary gland epithelial cells in a murine model of acute mastitis (Gonen et al., 2007) and that it is able to induce apoptosis and proliferation of bovine mammary cells (Long et al., 2001). These data suggest that *E. coli* P4 may possess some specific properties or genes that, in addition to LPS, allow it to induce mastitis.

Identification of these potential virulence genes requires a detailed analysis of the genome of *E. coli* P4 but also of its transcriptome and of its proteome, especially during infection. This work was undertaken to initiate the genetic characterization of *E. coli* P4. As a first

Figure 3. Predicted AA sequence of the protein encoded by the ORF4-argW of *Escherichia coli* P4. The double arrows indicate some of the conserved consensus motifs specific to the Sir2 proteins.

step, we identified to which of the 4 main phylogenetic groups of the *E. coli* species (namely, groups A, B1, B2, and D; Selander et al., 1987; Herzer et al., 1990) strain P4 belongs. Our data unambiguously demonstrate that *E. coli* P4 belongs to the phylogenetic group A. This group, like phylogenetic group B1, contains mainly commensal isolates, whereas groups B2 and D encompass intestinal and other extraintestinal pathogenic isolates (Picard et al., 1999). When analyzed in more detail, our result showed that the core genome of *E. coli* P4 is close to that of *E. coli* K-12 MG1655, a commensal strain. Indeed, all but 6 partial sequences of housekeeping genes of *E. coli* P4 shared 100% identity with those of *E. coli* K-12 MG1655. For 5 out of the 6, we observed that their sequences remained closest to those of *E. coli* K-12 MG1655. Because the main habitat of this species is the intestine of humans or warm-blooded animals, the environment of cows is likely to contain numerous fecal *E. coli* strains. In addition, fecal and mammary pathogen strains belong to similar serotypes (Burvenich et al., 2003). It is therefore not surprising that *E. coli* P4 belongs to a phylogenetic group that includes

numerous commensal strains and that its core genome is close to that of a commensal strain such as *E. coli* K-12 MG1655. Thus, these data support the hypothesis that *E. coli* P4, like other strains of *E. coli* isolated from mastitis cases, is an environmental pathogen that provokes transient infection of the mammary gland.

We refined this initial characterization, based on the core genome of *E. coli* P4, by characterizing genes belonging to its accessory gene repertoire, the set of genes likely to be responsible for particular properties of *E. coli* P4. In particular, it has been reported that, under specific conditions (toll-like receptor 4 knockout mice), *E. coli* P4 is capable of adhering to and invading epithelial cells of mammary gland in a murine model of acute mastitis, although the anatomy of the murine mammary gland is completely different from that of the bovine (Gonen et al., 2007), and of inducing apoptosis and proliferation of bovine mammary cells (Long et al., 2001). Using PCR, we tried to identify in *E. coli* P4 virulence factors that are present in other intestinal or extraintestinal pathogenic strains of *E. coli*. This approach allowed us to detect only the TraT factor, which

is involved in serum resistance (Montenegro et al., 1985). This component has been frequently identified in the mammary pathogen strains of *E. coli* (Kaipainen et al., 2002) and is thought to be involved in the protection of strains against the action of phagocytes. None of the other virulence factors we tried to detect, especially the 12 factors known to be involved in the process of cell adhesion, were observed (data not shown). These results indicate that either *E. coli* P4 is an environmental strain devoid of any specific virulence properties or genes or that it contains genes yet to be identified as such in a pathogenic *E. coli* strain. This second hypothesis led us to search for GEI in *E. coli* P4, knowing that specific virulence genes are often clustered into GEI (Dozois and Curtiss, 1999).

Foreign acquired DNA sequences are frequently integrated downstream from tRNA genes (Hacker and Kaper, 2000). In this way, numerous GEI have been found and characterized in commensal, intestinal, and extraintestinal pathogenic *E. coli* strains, revealing novel properties such as the tropism of these strains (Shpigel et al., 2008). Considering these data, as well as the properties of *E. coli* P4 and the absence of virulence factors commonly found in other pathogenic strains, we decided to seek potential GEI downstream of tRNA genes of this strain. We found that the genomic context of 7 tRNA genes differed from that observed in *E. coli* K-12 MG1655. These altered loci are *argW*, *asnT*, *aspV*, *leuX*, *pheV*, *serU*, and *thrW*. They correspond to loci where GEI are frequently detected, as in *E. coli* strain 536 (Oelschlaeger et al., 2002), in the avian pathogenic *E. coli* Ec222 strain (Parreira and Gyles, 2003), in *E. coli* RW1473 strain (Jores et al., 2001), and in the nonpathogenic probiotic *E. coli* strain Nissle 1917 (Grozdanov et al., 2004).

Sequence analysis of *argW*, *asnT*, *leuX*, *pheV*, *thrW* and *serU* loci showed that their immediate downstream region contained ORF encoding integrases that shared high percentages of similarity with integrases present at the same locus in other strains of *E. coli*. The presence of integrases downstream of a tRNA gene is a typical feature of GEI, suggesting that GEI are present in the genome of *E. coli* P4 downstream of these 6 tRNA genes.

The GEI present downstream of *argW* and *serU* were studied in more detail. The complete characterization of the altered *argW* locus revealed typical features of GEI: this GEI is flanked by 23-bp repeats, its size exceeded 10,000 bp (11,253 bp), it contained 2 ORF encoding mobile genetic elements (1 putative integrase and 2 putative resolvase), and its G+C content (40.92%) was clearly different from that of the complete genomes

of *E. coli* (about 50%). The GEI located at *serU* was partially sequenced: it contained 7 ORF, some of which showed interesting features (see below).

The presence of GEI in *E. coli* P4 raises the question about their role in the capacity of the strain to induce mastitis. Answering this question requires us to determine whether these GEI contain specific genes that could be involved in the pathogenicity of *E. coli* P4. Only the determination of the complete nucleotide sequence of the regions situated downstream from *asnT*, *leuX*, *pheV*, *serU*, and *thrW* loci would allow us to elucidate their exact genetic content, as was the case for the *argW* locus. The sequence data of the GEI we detected revealed the presence of several genes of interest. First, ORF1 of the *serU*-GEI encodes a protein similar to a bacteriophage protein implicated in O-antigen conversion. The O-antigen is responsible for the resistance of strains to phagocytosis. Consequently, it could be interesting to determine whether ORF1 is implicated in the conversion of the O-antigen of the strain *E. coli* P4. The second ORF worth mentioning is ORF4-*argW*, which has no homology with any ORF identified to date in any *E. coli* genomes. This ORF encodes a putative protein that contains a Sir2-like conserved domain; that is, a putative protein that catalyzes NAD⁺-dependent protein/histone deacetylation. Acetylation/deacetylation is one of the important regulation processes of cellular metabolism (Kouzarides, 2000). Sir2 proteins, also known as sirtuins, are found in all eukaryotes and many archaea and prokaryotes and have been shown to regulate gene silencing, DNA repair, metabolic enzymes, and life span (North and Verdin, 2004). To date, sirtuins of yeast and human have been extensively studied. The physiological functions and molecular interactions associated with the other sirtuins, especially the bacterial ones, are still partial. Considering the different roles that could be played by these proteins, it would be interesting to determine whether the protein encoded by the ORF4-*argW* could be involved in the pathogenesis of *E. coli* P4 by inducing, for example, transcriptional silencing, a phenomenon shown to play a critical role in bacterial virulence (Dagberg and Uhlin, 1992) or by being involved in a resistance mechanism to the oxidizing stress, which is particularly predominant in the mammary gland during a mastitis (Burvenich et al., 2003). To conclude, the disruption of these genes, followed by studies of the capacity of the mutants of inducing mastitis in animal, could be valuable to determine whether they are involved in the capacity of the strain to provoke mastitis. In addition to the complete characterization of the GEI of *E. coli* P4, it would be interesting to extend the study

we report here to determine the prevalence of DNA sequences identified in this report in strains isolated from mastitis cases

CONCLUSIONS

In conclusion, this paper reports the presence of GEI in a bovine mastitis pathogen strain of *E. coli*. The first results of sequencing for some loci and the complete characterization of one GEI revealed interesting features that distinguish the mastitis pathogen strain *E. coli* P4 from the commensal strain *E. coli* K-12 MG1655, even though its core genome is close to that of the commensal strain. The presence in the GEI of ORF that are present in other pathogenic strains of *E. coli* showed a connection between the genomes of *E. coli* P4 and other *E. coli* pathogenic strains. The systematic search for GEI in other *E. coli* mastitis pathogens displaying different behaviors during mastitis, their sequencing, and the study of effects of their disruption on the capacity of mutants of inducing mastitis might be a promising way to elucidate the pathogenesis of these strains. Further, such searches might allow us to determine whether the *E. coli* mastitis pathogens constitute a new pathotype in the *E. coli* species; that is, a group of mammapathogenic strains, as suggested by others (Shpigel et al., 2008).

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