Comparative Genomics between Two *Xenorhabdus bovienii* Strains Highlights Differential Evolutionary Scenarios within an Entomopathogenic Bacterial Species

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Abstract

Bacteria of the genus *Xenorhabdus* are symbionts of soil entomopathogenic nematodes of the genus *Steinernema*. This symbiotic association constitutes an insecticidal complex active against a wide range of insect pests. Within *Xenorhabdus bovienii* species, the *X. bovienii* CS03 strain (*Xb* CS03) is nonvirulent when directly injected into lepidopteran insects, and displays a low virulence when associated with its *Steinernema* symbiont. The genome of *Xb* CS03 was sequenced and compared with the genome of a virulent strain, *X. bovienii* SS-2004 (*Xb* SS-2004). The genome size and content widely differed between the two strains. Indeed, *Xb* CS03 had a large genome containing several specific loci involved in the inhibition of competitors, including a few NRPS-PKS loci (nonribosomal peptide synthetases and polyketide synthases) producing antimicrobial molecules. Consistently, *Xb* CS03 had a greater antimicrobial activity than *Xb* SS-2004. The *Xb* CS03 strain contained more pseudogenes than *Xb* SS-2004. Decay of genes involved in the host invasion and exploitation (toxins, invasins, or extracellular enzymes) was particularly important in *Xb* CS03. This may provide an explanation for the nonvirulence of the strain when injected into an insect host. We suggest that *Xb* CS03 and *Xb* SS-2004 followed divergent evolutionary scenarios to cope with their peculiar life cycle. The fitness strategy of *Xb* CS03 would involve competitor inhibition, whereas *Xb* SS-2004 would quickly and efficiently kill the insect host. Hence, *Xenorhabdus* strains would have widely divergent host exploitation strategies, which impact their genome structure.

Key words: entomopathogenic bacteria, insect, nematode, competitor inhibition, virulence.

Introduction

Bacteria are found in all environments, from the soils to the inside of eukaryotic cells. Selective pressures exerted by the ecological niche shape the size and the content of bacterial genomes. For example, host-associated bacteria, both mutualistic and parasitic, usually undergo genome reduction, due to several evolutionary forces. In nonobligate pathogenic bacteria, purifying selection leads to the inactivation of genes encoding proteins interfering with bacterial virulence (i.e., antivirulence genes) (Bliven and Maurelli 2012; Prosseda et al. 2012). In the nutrient-rich and stable host ecological niches of obligate intracellular bacteria, relaxed selective pressure often leads to the pseudogenization resulting in degradation and loss of metabolism and regulation genes. Moreover, the populations of highly host-restricted bacteria are usually very small. In this context, both genetic drift and reduced horizontal genetic transfer (HGT) opportunities bias genomic evolution toward reduction (Ochman and Moran 2001; Moran and Plague 2004; Ochman and Davalos 2006).

Environmental species, such as soil or marine bacteria, have to deal with nutrient-poor environments. If this environment is

stable, strong purifying selection favors minimization of cell and genome size, an evolutionary process named streamlining (Giovannoni et al. 2014). In contrast, the genome of bacteria living in changing and nutrient-poor environments contain several regulators, stress-resistance genes, and metabolism genes, which generates different sets of genes adapted to different sets of constraints (Ochman and Davalos 2006; Sanchez-Perez et al. 2008; Bratlie et al. 2010). Bacteria living in mixed-species environments also are subject to a strong competition in the nutrient exploitation. Genomes may hence harbor a great number of genes involved in secondary metabolism playing a role in synthesis of antimicrobial compounds. NRPS-PKS enzymes (nonribosomal peptide synthetases and polyketide synthases) have a central role in such metabolism (Challis and Hopwood 2003; Hibbing et al. 2010). As a consequence, within a given genus, the genomes from environmental bacteria are usually larger than the genomes of host-associated bacteria. As an example, unlike *Mycobacterium tuberculosis* or *Mycobacterium leprae*, *Mycobacterium marinum*, a marine bacterium and fish pathogen, has a large genome encoding several stress-resistance genes and a striking number of NRPS-PKS loci acquired during HGT events (Stinear et al. 2008).

How do genomes evolve when bacteria alternate between different ecological niches that exert opposite evolutionary pressures? The Enterobacteriaceae *Xenorhabdus nematophila* alternates between host-associated lifestyles, mutualistic, and pathogenic in the living invertebrate hosts, and in a mixed-species environment in the insect cadaver. Indeed, *X. nematophila* is an obligate symbiont of the entomopathogenic nematode *Steinernema carpocapsae*, which lives into the soils. The bacteria–nematode pair is pathogenic for a wide range of insects (Thomas and Poinar 1979; Herbert and Goodrich-Blair 2007). When the nematodes penetrate into the hemocoel (general cavity) of an insect larva, they release the *X. nematophila* bacteria from a specialized intestinal organ, the receptacle (Snyder et al. 2007; Chaston et al. 2013) into the insect hemolymph (equivalent of the blood). *Xenorhabdus nematophila* bacteria multiply into the hemolymph and kill the insect by septicemia and toxemia (Boemare 2002). The bacteria then degrade the insect tissues, enabling nematode maturation, and reproduction, while competing with soil microorganisms for nutrient acquisition in the insect cadaver. When the nutrient resources are depleted, the juvenile nematodes switch to the infective juvenile (IJ) stage, which reassociates with the bacteria, leaving the cadaver in search for a new host (Poinar 1990; Goodrich-Blair and Clarke 2007; Stock and Goodrich Blair 2008). Under laboratory conditions, the direct injection of *X. nematophila* into the hemocoel kills the insect host within 30 h (Sicard et al. 2004; Herbert and Goodrich-Blair 2007). The genome of *X. nematophila* consists of a medium-sized chromosome (4.43 Mb) and a 155 kb megaplasmid, and encompasses many genes potentially involved in the synthesis of insecticidal and in antimicrobial compounds (Ogier et al. 2010; Chaston et al. 2011). Abundant signs of recent genomic rearrangements suggest that alternation of ecological niches maintains an elevated genomic plasticity (Ogier et al. 2010).

In the same genus, the bacterial species *Xenorhabdus poinarii* displays similar cyclic and alternative way of life, but it is attenuated in virulence when directly injected into the insect hemolymph (Akhurst 1986; Ogier et al. 2014). Interestingly, the *X. poinarii* strain G6 harbors only a small 3.66 Mb chromosome and displays decay of isolated genes and excision of some genomic loci. These typical features of attenuated free pathogenic bacteria or facultative mutualistic bacteria growing exclusively within hosts are general traits for the whole species *X. poinarii* (Ogier et al. 2014). Therefore, the evolutionary scenario in the *X. poinarii* species is likely a reductive genomic evolution, the result of a high dependence on the associated nematode for some virulence-related functions and of a more advanced process of host restriction (Ogier et al. 2014).

In this study, we focused on the *Xenorhabdus bovienii* species, whose biological features are less homogeneous than the *X. nematophila* and *X. poinarii* ones. First, the monophyletic bacterial clade *X. bovienii* interacts with at least nine different nematode species from the *Steinernema* genus, belonging to two phylogenetic clades (Lee and Stock 2010). Second, the *Steinernema* spp.—*X. bovienii* pairs and the bacteria alone show highly variable virulence in lepidopteran insects (Bisch et al. 2015). Nevertheless, recent genomic analyses confirm the species status of the *X. bovienii* clade according to current standard criteria in microbial systematics (Murfin et al. 2015). These data suggest different evolutionary pressures within the *X. bovienii* species. Two *Steinernema* spp.—*X. bovienii* pairs have been recently studied. The *Steinernema jollieti*/*X. bovienii* strain SS-2004 pair was isolated in United States (Spiridonov et al. 2004). The 4.23 Mb chromosome of *X. bovienii* SS-2004 (*Xb* SS-2004) has been sequenced and shares numerous features with the *X. nematophila* one (Ogier et al. 2010; Chaston et al. 2011). The *Steinernema weiseri*—*X. bovienii* strain CS03 pairs was isolated in Czech Republic (Mráček et al. 2003). Both the bacteria–nematode pair and the bacteria alone show attenuated virulence in lepidopteran insects (Bisch et al. 2015). The bacteria–nematode pair and the bacteria alone displayed attenuated virulence and no virulence, respectively, in lepidopteran insects (Bisch et al. 2015). This suggests that the nematode *S. weiseri* is responsible for certain virulence functions, whereas, in other *Steinernema–Xb* associations, the bacteria are virulent on their own.

In this study, we sequenced the *X. bovienii* CS03 (*Xb* CS03) genome. We compared the genomes of *Xb* SS-2004 and *Xb* CS03. Considering the differences in genome size, and in pseudogene and gene content between the two strains, we suggest that *Xb* SS-2004 specializes in exploitation of the insect host, whereas *Xb* CS03 inhibits its competitors.

Materials and Methods

Bacteria, Nematodes, and Growth Conditions

Xb CS03 was isolated in Czech Republic (Europe) from the *Steinernema weiseri* 583 nematode (Mráček et al. 2003; Tailliez et al. 2006). The *Xb* SS-2004 used for the genome comparison was isolated in Missouri (Northern America) from the *Steinernema jollieti* nematode (Spiridonov et al. 2004; Chaston et al. 2011). *Xb* strains were grown at 28 °C. *Serratia marcescens*, *Corynebacterium xerosis*, *Bacillus megatherium*, *Enterobacter cloacae*, *Micrococcus luteus*, *Ochrobactrum intermedium*, *Proteus vulgaris*, and *Pseudomonas putida* were grown at 37 °C. All bacteria were routinely grown in Luria–Bertani (LB) broth or on nutrient agar (Difco). Kanamycin was added at 40 μg/ml when needed. Bacterial strains were stored at −80 °C with 16% glycerol (vol/vol).

Xenorhabdus Phenotype Assays

The antimicrobial activity of *Xb* CS03 and *Xb* SS-2004 against bacterial strains and their hemolytic activity were assessed as previously described by Boemare et al. (1997). Briefly, to assess antimicrobial activity, *Xenorhabdus* bacteria were grown for 48 h on nutrient ager plates and then killed by exposing the plates to chloroform for 20 min. The plates were left for 10 min under a sterile air flow to allow the chloroform to evaporate. Overnight cultures of bacteria were diluted (20%; OD_{540nm} ~ 0.1) in sterile soft agar (nutrient broth, 5.0 g; bacto-agar, 7.0 g; water, 1L) and poured onto the prepared plates. When *Xenorhabdus* and *Photorhabdus* strains, which are killed by warm sterile soft agar, were used, overnight cultures were diluted in LB broth. The diameter of the inhibition zone was recorded after 24 h of incubation at 37 °C (environmental bacteria) or 28 °C (*Xenorhabdus* and *Photorhabdus*). To assess their hemolytic activity, the *Xenorhabdus* bacteria were grown on trypticase soy (bioMérieux) with 5% (vol/vol) defibrinated sheep blood (bioMérieux). Hemolysis was defined as the observation of a clear halo around bacteria grown on standard sheep blood agar plates.

Sequencing and Assembly of *Xb* CS03 Genome

The complete genome of *Xb* CS03 was sequenced as previously described (Ogier et al. 2014) with 6531 Sanger reads, a 20-fold coverage of 454 GSflx reads, a 14-fold coverage of mate-paired 454 GSflx reads (library insert around 3 kbases) and a 110-fold coverage of Illumina reads (36 bp). For the finishing phases, primer walking of clones, polymerase chain reactions (PCRs), and in vitro transposition technology was used generating 844, 286, and 3,028 additional reads.

Genomic Analysis

Functional annotation of the *Xb* CS03 genome and analysis of the regions of genomic plasticity (RGP) of *Xb* CS03 and *Xb* SS-2004 were carried out as previously described (Ogier et al. 2010, 2014). We used the RGPFinder web tool implemented on the MaGe annotation platform to identify genomic regions displaying breaks in synteny between the *Xb* CS03 and *Xb* SS-2004 genomes. If the regions displayed characteristics typical of foreign DNA acquired by HGT, such as compositional bias (GC% deviation, codon adaptation index) or tRNA, IS, integrase genes, and genetic elements involved in DNA mobility, they were classified as genomic islands (GI) or prophages (P), if identified as such by Prophinder (Lima-Mandez et al. 2008). Regions without such features were classified as $RGP_{sensu\ stricto}$. We used the SiLiX program (Miele et al. 2011) of the MicroScope platform (Vallenet et al. 2013) to describe the pan-genome, core genome, and flexible genomes inside the *Xenorhabdus* genus (homology constraints: 50% amino acid identity and 80% alignment coverage) or inside the *Xb* species (homology constraints: 80% amino acid identity and 80% alignment coverage). Strain-specific genes were manually classified according to their annotations.

Pseudogene Detection

Pseudogenes were identified using the Gene Fission tool of the MicroScope platform. This tool provides a list of candidate genes potentially involved in a fission event (predicted pseudogenes). Fission events are computed from the synteny results obtained from the genomes available in the PkGDB database. Predicted pseudogenes were manually inspected to remove false positives on the basis of the gene size and identity compared to reference sequences. Pseudogenes were classified as specific (i.e., pseudogenized only in the reference genome) or nonspecific (i.e., pseudogenized in both *Xb*). Functional categories were manually assigned to pseudogenes according their annotation.

NRPS-PKS Genes

NRPS-PKS loci were detected using the "2metDB" method (Bachmann and Ravel 2009) implemented in the MicroScope platform and manually curated on the basis of their annotation. We considered that a NRPS/PKS was conserved in *Xenorhabdus* and other bacterial species when they shared ≥50% amino acid identity. A locus was considered complete if the alignment coverage was at least 80% and partial otherwise.

Reverse Transcription-PCR

RNA extraction and cDNA synthesis were performed as described by Jubelin et al. (2013). Briefly, total RNA was extracted from a stationary phase culture (OD_{540nm}~ 1.5) of *Xb* CS03 with an RNeasy Protect Bacteria miniprep kit

(Qiagen), including DNase I incubation, in accordance with the manufacturer's recommendations. The absence of DNA contamination was checked by carrying out a control amplification of the 16S rRNA gene. The quantity and quality of total and messenger RNA were assessed with a NanoDrop 2000 spectrophotometer (Thermo Scientific) and an Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent), respectively. The cDNA was synthesized from 1 μg of total RNA, with Super Script II Reverse Transcriptase (Invitrogen) and random hexamers (100 ng.μl^{-1}, Applied Biosystems).

The primers used to amplify each *xaxAB* fragment are listed in supplementary table S1, Supplementary Material online. The PCR on *Xb* CS03 cDNA was realized with Taq polymerase (Invitrogen) according to the manufacturer recommendations, using a hybridization temperature of 57 °C and an elongation time of 90 s.

Construction of Recombinant *Xb* CS03

A XbaI-SalI fragment containing the *xaxAB* from *X. nematophila* F1 was hydrolyzed from the pBB*xaxAB* plasmid built in a previous study (Vigneux et al. 2007). The *xaxAB* fragment was then ligated into the medium copy plasmid pBBR1-MCS2 (Kovach et al. 1995) hydrolyzed with SalI and XbaI. The resulting plasmid, pBBMCS2-*xaxAB*, was checked by sequencing (Millegen, Labège, France). In this construction the *xaxAB* locus is under the control of the P_{lac} promoter, whose transcriptional activity is constitutive in *Xenorhabdus*. Finally, pBBMCS2-*xaxAB* was introduced into *Xb* CS03 by mating as described in Givaudan and Lanois (2000).

Results

General Genomic Features of *Xb* CS03

Xb CS03 contains a 4,635,301 bp chromosome encoding 4,757 coding sequences (table 1). This is the largest chromosome described for a completely assembled *Xenorhabdus* strain to date, the size of the other chromosomes ranging from 3,659,522 to 4,432,590 bp (Chaston et al. 2011; Ogier et al. 2014). *Xb* CS03 also contains a 177 kb megaplasmid and an 8 kb plasmid (supplementary table S2, Supplementary Material online). In the *Xb* CS03 plasmid, 8 out of the 11 coding sequences are identical to coding sequences from the *Xenorhabdus doucetiae* plasmid (Ogier et al. 2014). *Xb* CS03 megaplasmid harbors 197 coding sequences, some of which encoding putative extracellular enzymes, putative antibiotic resistance cassettes, toxin–antitoxin systems, and NRPS/PKS. This content is largely different from the *X. nematophila* ATCC 19061 megaplasmid previously described (Chaston et al. 2011). The *repA* gene of the *Xb* CS03 megaplasmid (XBW1_mp0001) is homologous to the RepFIB replicon present in the large enterobacterial plasmids of the IncF and IncP incompatibility groups (Gibbs et al. 1993). In contrast, the *repA* gene of the *X. nematophila* megaplasmid (XNC1_p0159) is homologous to the RepAC replicon from plasmids belonging to the IncA/C incompatibility group (Llanes et al. 1996). These data indicate that these two plasmids have different origins. In contrast, *Xb* CS03 megaplasmid shares 61, 57, and 44 orthologous coding sequences with the chromosome of *X. nematophila* ATCC19061, *Xb* SS-2004, and *Xb* CS03, respectively (best bidirectional hit, 35% amino acid identity, 80% alignment coverage). This suggests an elevated intragenomic flow between megaplasmid and chromosome, rather than an entire megaplasmid horizontal genetic transfer between *Xenorhabdus* strains.

We previously compared four *Xenorhabdus* genomes. Pan and core genomes were composed of 7,250 and 1,904 gene families, respectively (Ogier et al. 2014). The addition of the *Xb* CS03 genome in this analysis (homology constraints: 80% amino acid identity and 80% alignment coverage) does not modify drastically the size of the *Xenorhabdus* core genome (1,673 gene families; fig. 1). However, the pan genome size increased with the *Xb* CS03 genome (10,600 gene families; fig. 1), which confirms the diversity of the flexible genome (pan genome minus core genome) of the *Xenorhabdus* genus (Ogier et al. 2010).

Genomic Comparison within the Xb Species: General Features

Comparison of general genomic features between *Xb* CS03 and *Xb* SS-2004 genomes highlights two main differences (table 1). The *Xb* SS-2004 genome is slightly richer (24%) in insertion sequences than the *Xb* CS03 genome, and the number of pseudogenes in the *Xb* CS03 genome is 52% higher than in the *Xb* SS-2004 genome. Interestingly, among the 99 *Xb* CS03 pseudogenes, 40 matched with intact genes from *Xb* SS-2004, whereas among the 65 *Xb* SS-2004 pseudogenes, only 17 matched with intact genes in *Xb* CS03 (supplementary table S3, Supplementary Material online). Hence, while the pseudogenes of *Xb* SS-2004 mainly belong to the *Xb* core-genome (i.e., degraded also in *Xb* CS03), 40% of *Xb* CS03 pseudogenes are strain-specific. We classified the pseudogenes into nine functional categories (Cell division, DNA/RNA metabolism, Host/Environment interaction, Metabolism, Phage/Recombination, Protein biosynthesis, Regulation, Transport, and Unknown function) (fig. 2). *Xb* SS-2004 is particularly rich in pseudogenes from the "Phage/Recombination" category (32% of the pseudogenes). Phages and transposases may undergo a selective pressure which leads to their pseudogenization and deletion in bacterial genomes (Lawrence et al. 2001). This phenomenon could account for the pseudogenization of such genes in *Xb* SS-2004. However, the main difference is in the "Host and Environment Interaction" category, which represents 39% of the total pseudogenes content in *Xb* CS03 and only 26% of the *Xb* SS-2004 pseudogenes.

Table 1

Genome Features of *X. bovienii* CS03 (*Xb* CS03) Compared with *X. bovienii* SS-2004 (*Xb* SS-2004)

	Xb CS03			*Xb* SS-2004
Feature	***Chromosome***	***Megaplasmid***	***Plasmid***	***Chromosome***
Size (bp)	4,635,301	177,250	8,117	4,225,498
G+C content (%)	44.77	43.89	43.87	44.97
Coding sequences	4,757	197	11	4,362
Coding density (%)	83.63	82.82	62.95	84.92
Average CDS length (bp)	843.62	799	502.36	843.05
Average intergenic length (bp)	162.37	145.51	422	158.16
Repeated regions (%)	14.69	7.24	0	16.27
Pseudogenes	99	3	0	65
Insertion sequences[a]	298	29	0	369
Phagic genes[b]	454	119	0	450
Prophage loci[b]	10	1	0	8
rRNA operons	7	0	0	7
tRNAs	76	0	0	83
Accession number	FO818637	FO818638	FO818639	FN667741

[a]identified with the IS Saga tool (Varani et al. 2011).
[b]identified with Prophinder (Lima-Mendez et al. 2008).

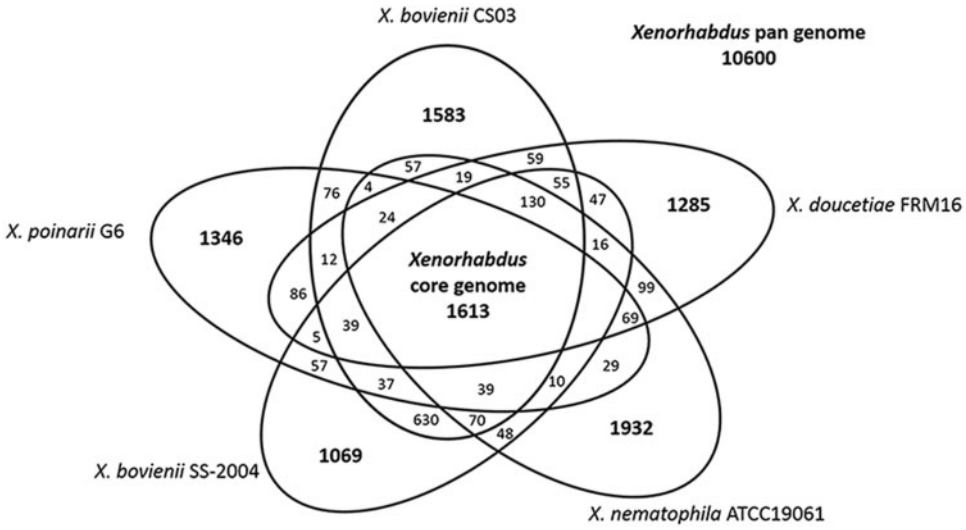

Fig. 1.—Venn diagram showing numbers of shared gene families (80% amino acid identity, 80% alignment coverage) in the genomes of *X. bovienii* CS03, *X. bovienii* SS-2004, *X. nematophila* ATCC19061, *X. doucetiae* FRM16, and *X. poinarii* G6.

Xb Core and Flexible Genome

We compared the *Xb* CS03 and *Xb* SS-2204 genomic contents. The *Xb* core-genome and the *Xb* specific core-genome (which excludes the genes also found in *X. nematophila* ATCC19061, *X. doucetiae* FRM16 and *X. poinarii* G6) were composed of 2,673 and 1,000 genes, respectively (fig. 3A and supplementary table S4, Supplementary Material online). *Xb* CS03 and *Xb* SS-2004 flexible genomes contained 1,815 and 1,267 strain-specific genes, respectively (fig. 3A and supplementary table S4, Supplementary Material online).

We examined the structure of *Xb* CS03 and *Xb* SS-2004 flexible genome by searching for RGP. These regions were identified as GI, prophage loci (P), or uncharacterized regions ($RGP_{sensu\ stricto}$), according to the criteria used in a previous study (Ogier et al. 2010, 2014). We identified more RGP in *Xb* CS03 flexible genome (69 chromosomal RGP encompassing 14 GI, 10 P, and 45 $RGP_{sensu\ stricto}$; 25% of the genome) than in *Xb* SS-2004 flexible genome (55 chromosomal RGP encompassing 11 GI, 8P, and 36 $RGP_{sensu\ stricto}$; 20% of the genome; supplementary table S5, Supplementary Material online). One additional prophage was located on the *Xb* CS03 megaplasmid. We identified one of the *Xb* CS03 prophages (XBW1_1585-1616) as similar to the xenorhabdicin-encoding phage *xbp1* of *Xb* SS-2004 (Morales-Soto et al. 2012) and named it *xbwp1* (supplementary tables S5 and S6, Supplementary Material online). One or several of the RGP

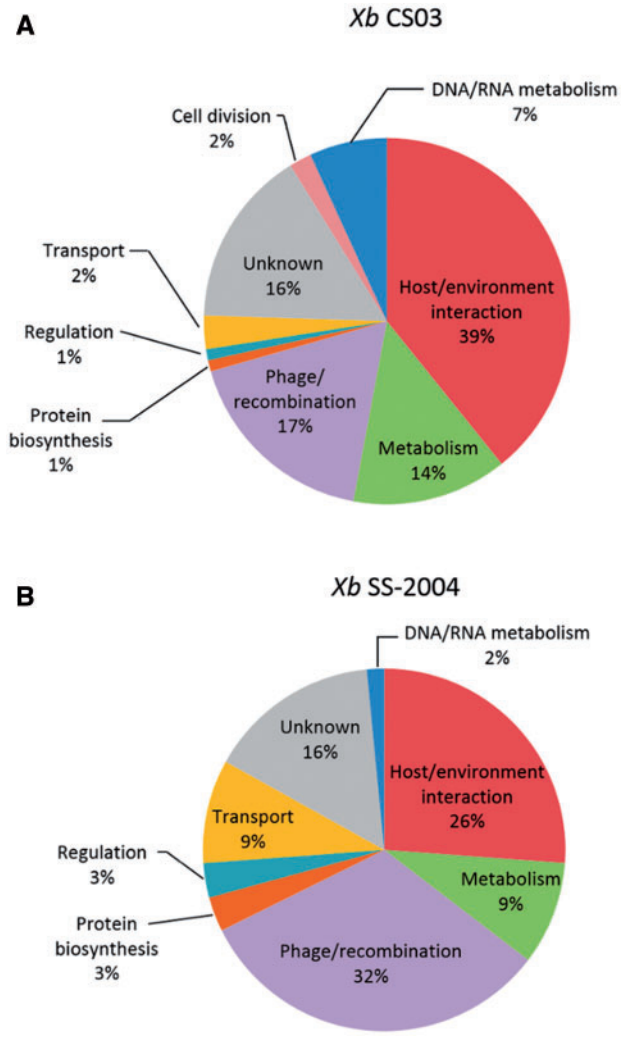

Fig. 2.—Functional classification of pseudogenized genes in *X. bovienii* CS03 and *X. bovienii* SS-2004. (*A*) Functional classification of *X. bovienii* CS03 pseudogenes. (*B*) Functional classification of *X. bovienii* SS-2004 pseudogenes.

may be embedded in an integrative conjugative element (ICE; (Ogier et al. 2010; Guglielmini et al. 2011). Four ICEs were identified in *Xb* CS03, whereas two ICEs had previously been described in *Xb* SS-2004 (Chaston et al. 2011; Ogier et al. 2014). Only ICE1 was complete (supplementary table S7, Supplementary Material online). In summary, these results show that mobile genetic elements make up a large proportion of the flexible gene content of *Xb* CS03, substantially contributing to its larger genome size compared with *Xb* SS-2004. Classification of the flexible gene content into the functional categories defined above resulted in similar distributions for *Xb* CS03 and *Xb* SS-2004 with a slight difference in the "Host/Environment interaction" and "Unknown function" classes (fig. 3B).

The *Xb* CS03 genome therefore displays two remarkable features by comparison with *Xb* SS-2004: a large flexible genome and an important content of pseudogenes specific to *Xb* CS03 (belonging to the flexible genome). Furthermore, the coding sequences that underwent pseudogenization in the *Xb* CS03 genome are frequently annotated as potentially involved in the host and environment interactions. We thus focused our follow-up analyses on the flexible gene content falling into the functional class Host and environment interactions.

Xb CS03 Wealth in Antimicrobial Compounds Synthesis Loci Correlates with a Strong Antimicrobial Activity

The nature of the flexible genome coding sequences classified in the Host and environment interaction category is largely different between *Xb* CS03 and *Xb* SS-2004 (fig. 3C and supplementary table S8, Supplementary Material online). First, *Xb* CS03 contains more coding sequence potentially involved in resistance to DNA invasion, such as restriction–modification systems for resistance to bacteriophage invasion (Kobayashi 2001) and toxin–antitoxin systems for resistance to mobile genetic elements, such as GIs and plasmids (Schuster and Bertram 2013).

The flexible genome of *Xb* CS03 is also particularly rich (42% of the coding sequences) in loci involved in the synthesis of secondary metabolites with potential antimicrobial activity (fig. 3C and supplementary table S8, Supplementary Material online). A major part of those loci encode NRPS/PKS (58% of the Host and environment interaction genes contained in the flexible genome of *Xb* CS03, vs. 10% in *Xb* SS-2004). Among the NRPS-PKS loci specific to *Xb* CS03, three (XBW1_0736-0742, XBW1_1481-1490, and XBW1_3836-3844) belonged to $RGP_{sensu\ stricto}$ (respectively, RGPs 12, 24, and 60) and one was an isolated gene (XBW1_3328). Those NRPS/PKS loci were present in other *Xb* strains as well as in other *Xenorhabdus* species (supplementary table S9, Supplementary Material online), which indicates that they could have been excised from the genome of *Xb* SS-2004.

We compared the antimicrobial activity of *Xb* CS03 and *Xb* SS-2004 against several potential bacterial competitors: *Xenorhabdus* and *Photorhabdus* strains, but also *P. vulgaris*, *Se. marcescens*, *C. xerosis*, *B. megatherium*, *E. cloacae*, *O. intermedium*, and *Ps. putida*, bacterial species that have been found in association either with the soil, with the insect *G. mellonella* or with entomopathogenic nematodes *Steinernema* (Lysenko and Weiser 1974; Boemare et al. 1983; Aguillera and Smart 1993; Gouge and Snyder 2006). *Micrococcus luteus* was used as reference strain to assess antimicrobial activities (Boemare et al. 1997). When tested against strains from the closely related genera *Xenorhabdus* and *Photorhabdus*, both *Xb* CS03 and *Xb* SS-2004 were able to inhibit bacterial growth, although the patterns of inhibition were different (table 2). When tested against phylogenetically distant bacteria, *Xb* CS03 displayed antimicrobial activity against more than half of the tested strains (*C. xerosis*,

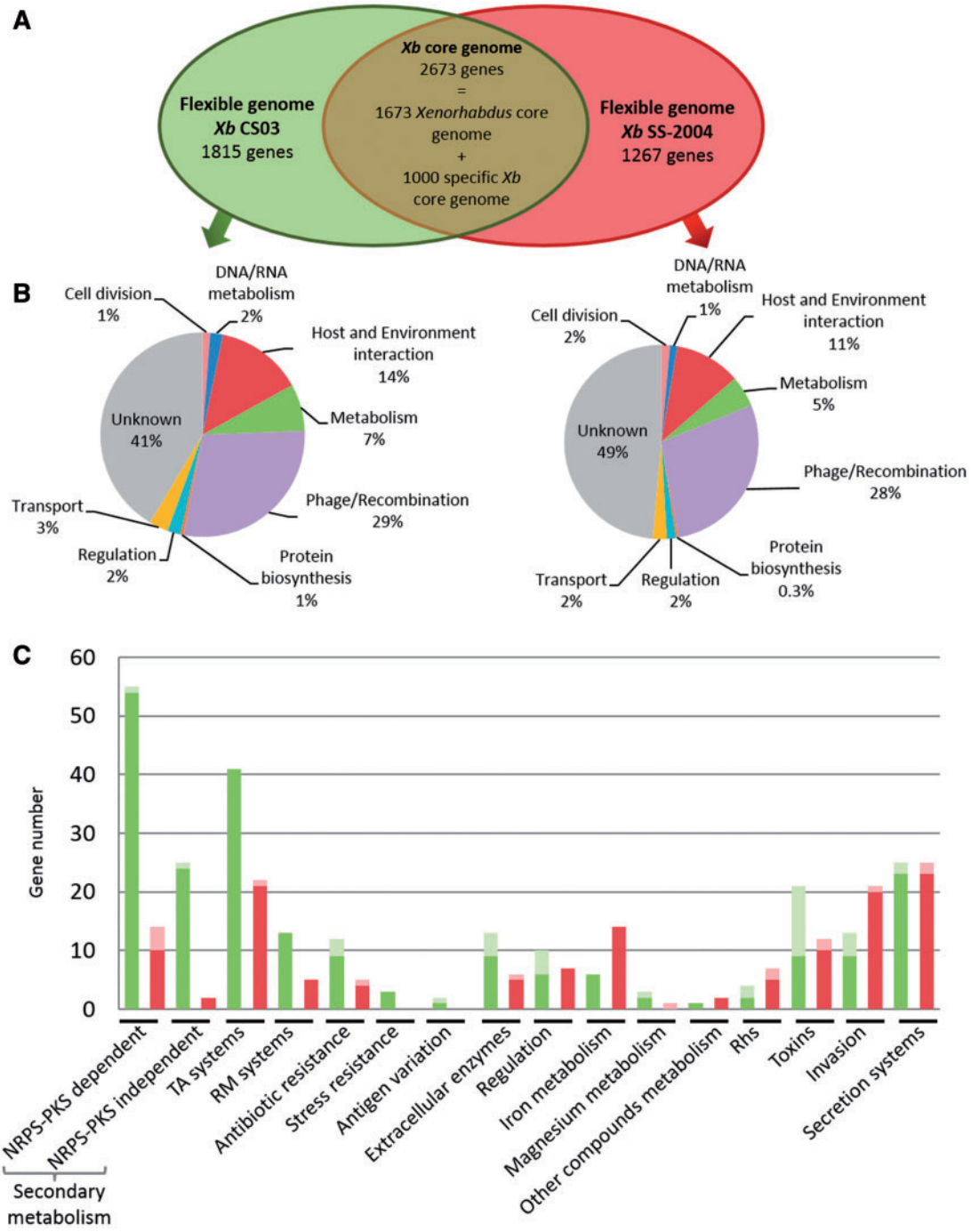

Fig. 3.—*Xenorhabdus bovienii* pan, core, and flexible genomes. (*A*) Venn diagram showing numbers of shared gene families (80% amino acid identity, 80% alignment coverage) in the genomes of *X. bovienii* CS03 and *X. bovienii* SS-2004. The *Xb* core genome (2,673 gene families) includes the *Xenorhabdus* core genome (1,673 gene families common to *X. nematophila* ATCC19061, *X. doucetiae* FRM16, and *X. poinarii* G6) and the specific *X. bovienii* core genome (1,000 gene families). (*B*) Functional classification of coding sequences in *X. bovienii* SS-2004 and *X. bovienii* CS03 flexible genomes. The coding sequences of each flexible genome have been classified into nine functional categories (Cell division, DNA/RNA metabolism, Host/Environment interaction, Metabolism, Phage/Recombination, Protein biosynthesis, Regulation, Transport, and Unknown function) according to their annotation. (*C*) Specific host and environment interaction genes functions for the *X. bovienii* SS-2004 (red) and *X. bovienii* CS03 (green) flexible genomes. The genes belonging to the flexible genome of *Xb* CS03 and *Xb* SS-2004 and encompassed within the Host and environment interaction category were classified according to their annotation. In the histogram, the pseudogenes are indicated in lighter color. RM: Restriction-Modification; TA: toxin-antitoxin; Rhs: Rearrangement hotspots.

B. megatherium, *E. cloacae*, *O. intermedium*, and *M. luteus*), whereas *Xb* SS-2004 was active only against the highly sensitive strain *M. luteus* (table 2).

Xb CS03 also contains several loci encoding non-NRPS-PKS enzymes, which may catalyze the production of antifungal metabolites (fig. 3C and supplementary table S8, Supplementary Material online). For example, a locus displaying similarity to subregions of the *Streptomyces tendae* nikkomycin synthesis locus (XBW1_3291-XBW_3307) was present only in *Xb* CS03 (fig. 4). Nikkomycin has antifungal activity against *Saccharomy cescerevisiae* and the pathogenic fungi *Candida albicans* and *Cryptococcus neoformans* (Cabib 1991; Hector 1993). In *Xb* CS03, the nikkomycin-like locus did not have the typical features of a GI, but was located in an $RGP_{sensu\ stricto}$ (RGP56). As the nikkomycin locus is absent from *Xb* SS-2004, but also from *X. nematophila* ATCC19061, *X. doucetiae* FRM16, and *X. poinarii* G6 genomes, it was probably acquired by the *Xb* CS03 genome.

Table 2

Antimicrobial Activity of *X. bovienii* CS03 and SS-2004 against Bacterial Species Associated with the Insect or the Nematode

	Xb CS03	*Xb* SS-2004
Xb CS03	0	0
Xb SS-2004	14–17	0
Xenorhabdus bovienii TR03	0	10–25
Xenorhabdus bovienii FR44	0	15–20
Xenorhabdus bovienii TB10	0	0
Xenorhabdus nematophila ATCC19061	15	16–18
Xenorhabdus doucetiae FRM16	0	0
Xenorhabdus poinarii G6	0	0
Photorhabdus luminescens TT01	28–30	0
Proteus vulgaris CIP 5860	0	0
Serratia marcescens	0	0
Corynebacterium xerosis	22–25[a]	0
Bacillus megatherium	41	0
Enterobacter cloacae	10–20	0
Micrococcus luteus	55	15
Ochrobactrum intermedium LMG 3301	0	0
Pseudomonas putida	10–15[a]	0
Stenotrophomonas maltophilia	0	0

Note.—At least two independent experiments were performed, except for *Bacillus megatherium* and *Stenotrophomonas maltophilia* (one experiment). Target strains come from our laboratory collection. Antimicrobial activity is given as the diameter of the clearing halo around the *X. bovienii* colony (in mm).

[a]Presence of colonies into the clearing halo.

Pseudogenization of Genes Encoding Putative Virulence Factors in the *Xb* CS03 Flexible Genome

At first glance, *Xb* CS03 flexible genome displayed less coding sequences involved in bacterial invasion and iron metabolism. Additionally, one could note that several *Xb* CS03 genes encoding transcriptional regulators but also extracellular enzymes, proteins involved in the host invasion, and toxins were pseudogenized (fig. 3C).

As a first example, *Xb* CS03 *tc* loci were shuffled, and either highly degraded or interrupted by transposases. Consequently, no complete *tc* locus was found in *Xb* CS03. By contrast, there were three and one whole *tc* locus (encoding A, B, and C components) in *X. nematophila* ATCC19061 and *Xb* SS-2004, respectively (fig. 5). The *tc* loci of *Xenorhabdus* encode families of insecticidal toxins conserved in several other entomopathogenic bacteria (Hinchliffe et al. 2010). Some proteins of the Tc complex display high oral toxicity in insects (Bowen et al. 1998), in which they disrupt the insect gut epithelium (Sheets et al. 2011) and inhibit the phagocytosis of insect hemocytes (Lang et al. 2010).

The *xaxAB* locus is also an interesting example of pseudogenization. This locus was previously described in *X. nematophila* and encodes the hemolysin XaxAB, a binary pore-forming toxin with apoptotic and necrotic activity toward insect cells and probably required during the

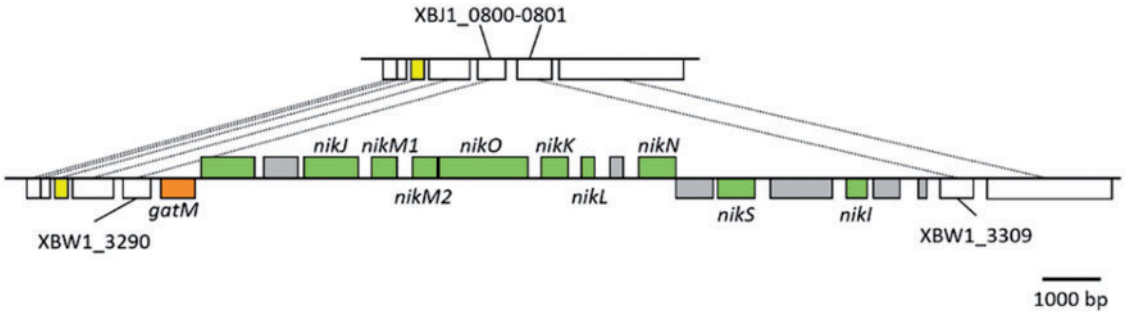

Fig. 4.—*Xenorhabdus bovienii* CS03-specific nikkomycin-like locus. The figure shows the nikkomycin-like locus of *Xb* CS03 (bottom), located between two genes of the core genome (XBW1_3290-3309). In the *Xb* SS-2004 genome (top), no genomic locus is inserted between the orthologous genes (XBJ1_0800-0801). The locus responsible for nikkomycin biosynthesis in *Streptomyces tendae* contains 22 genes (*nikA-V*). In *Xb* CS03, only subregions are present: the *nikIJKLMO* genes responsible for the synthesis of aminohexuronic acid, and the *nikS* gene encoding an enzyme responsible for the loading of the precursor of the hydroxypyridylhomothreonine amino acid to the bidomain protein NikT. Nikkomycin-like locus genes are shown in green, putative regulators in orange, transposases and insertion sequences in yellow, genes of unknown function in gray, and core-genome genes in white.

degradation of the insect cadaver in *X. nematophila* (Jubelin et al. 2011). In *Xb* SS-2004, the genomic locus *xaxAB* (XBJ1_1710-1711) displayed the same organization as in the *X. nematophila* genome and the products share, respectively, 74% and 69% of identity with the XaxA and XaxB proteins. In contrast, in *Xb* CS03, both genes were pseudogenized: *xaxA* (XBW1_1010-1032) was interrupted by a prophage insertion and *xaxB* (XBW1_1009-1010) was split in two fragments (fig. 6A). In vitro, XaxAB displays a hemolytic activity against sheep erythrocytes (Brillard et al. 2001). We tested the hemolytic activity of *Xb* CS03 and *Xb* SS-2004 on sheep red blood agar plates (fig. 6B). *Xb* CS03, contrarily to *Xb* SS-2004, did not display a hemolysis halo around the bacteria.

In order to test if the observed pseudogenization had an impact on the transcription of the gene remnants, we conducted reverse transcription-PCR with primers targeting each of the four remnants of the *Xb* CS03 *xaxAB* locus on stationary phase cultures. The 5′-fragments of both *xaxA* and *xaxB* remained transcribed, whereas the 3′-fragments were not (fig. 6C). To test the ability of *Xb* CS03 to express and secrete a functional XaxAB hemolysin, we introduced the pBBMCS2-*xaxAB* plasmid, which constitutively expresses the *xaxAB* locus from *X. nematophila* F1, in *Xb* CS03. The hemolytic activity of the *Xb* CS03/pBBMCS2- *xaxAB* transconjugant was complemented (fig. 6D). Altogether, these data suggest that the pseudogenization of the *xaxAB* locus is recent.

Discussion

The genomes of bacteria undergo changes due to the selective pressures exerted by their lifestyles, either host-associated or environmental. In this study, we explored the genome structure and content of *Xb* CS03. As in other *Xenorhabdus* strains, the life cycle of *Xb* CS03 alternates between close association with the nematode and the insect, and environmental steps in which it must compete with other microbial species. However, unlike many *Xenorhabdus* strains, *Xb* CS03 alone and *Xb* CS03 associated with its nematode *S. weiseri* were nonvirulent and displayed attenuated virulence, respectively, in lepidopteran larvae (Bisch et al. 2015).

Xb CS03 has the largest genome of any of the *Xenorhabdus* strains sequenced to date. Large genomes are generally observed in species or strains retaining a capacity for

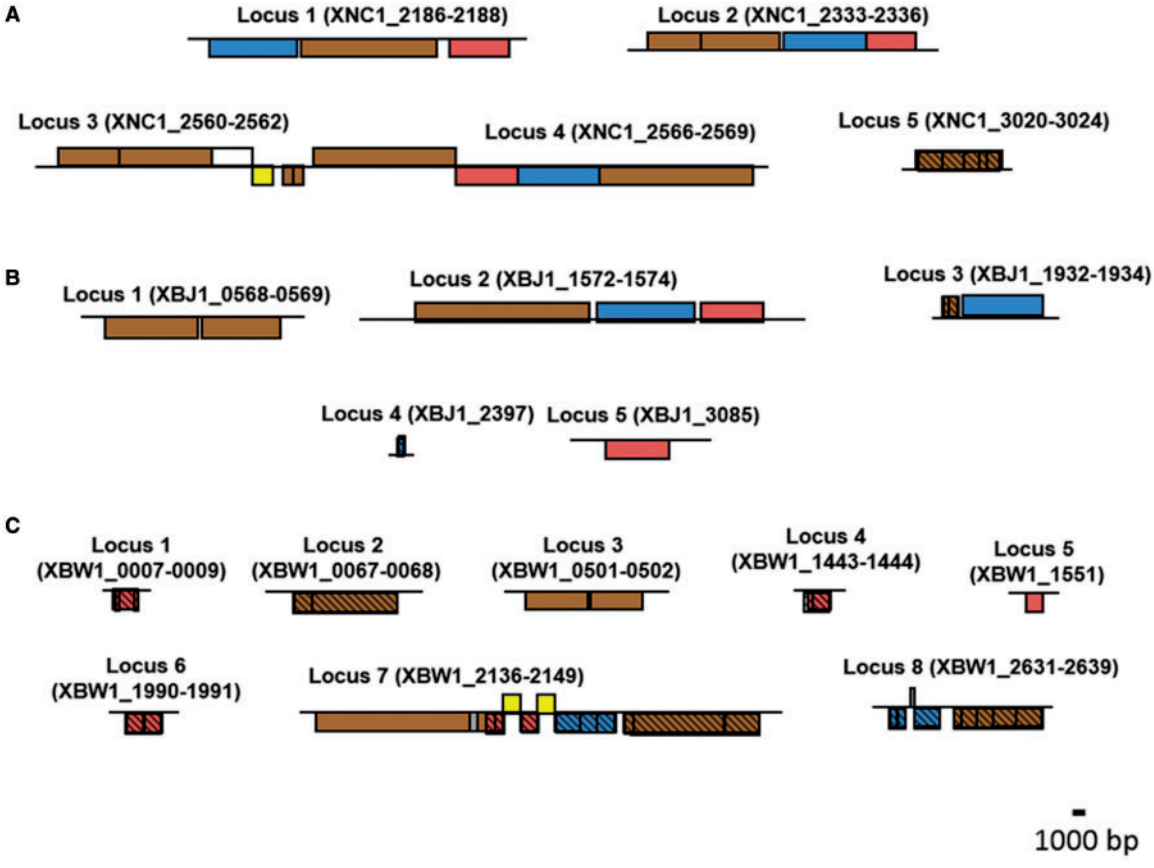

Fig. 5.—The *tc* toxin loci of *X. nematophila* ATCC19061 (*A*), *X. bovienii* SS-2004 (*B*), and *X. bovienii* CS03 (*C*). Genes encoding the A component of the Tc toxin are colored in brown, the B component in blue, the C component in red; transposases and insertion sequences are colored in yellow, genes of unknown function in gray, and core-genome genes in white. Gene fragments are indicated by hatching.

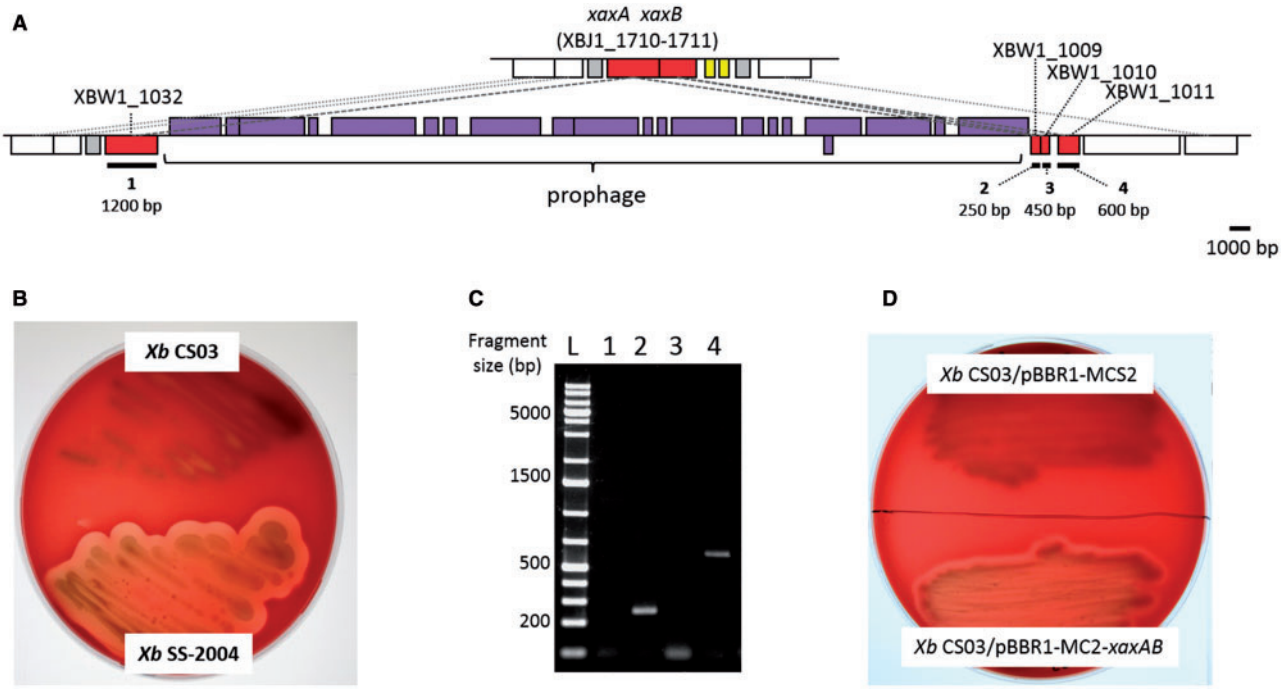

Fig. 6.—The *xaxAB* locus and the hemolytic activity of *X. bovienii* CS03. (*A*) The *xaxAB* loci of *X. bovienii* SS-2004 (top) and *X. bovienii* CS03 (bottom). For each *xaxAB* gene remnant of *X. bovienii* CS03, the size is indicated. The *xaxAB* genes are colored in red, the prophage in purple, transposases and insertion sequences in yellow, genes of unknown function in gray, and core-genome genes in white. (*B*) Hemolytic activity of *X. bovienii* SS-2004 (bottom) and *X. bovienii* CS03 (top). The indicated strains were streaked onto sheep red blood agar plates and incubated at 28 °C. Zones of clearing were observed over a 15-h period. (*C*) Electrophoretic separation of the products of reverse transcription-PCR on gene remants of the *X. bovienii* CS03 *xaxAB* locus. The amplified fragments were separated on a 1% agarose gel. The lane numbers correspond to the fragments described in figure 6*A*. Lane 1: 3′- fragment of *xaxA*; lane 2: 5′-fragment of *xaxA*; lane 3: 3′-fragment of *xaxB*; lane 4: 5′-fragment of *xaxB*. (*D*) Hemolytic activity of *X. bovienii* CS03 pBBR1-MCS2 (top) and *X. bovienii* CS03 pBBMCS2-*xaxAB* (bottom). Aliquots (20 µl) of cultures of the indicated strains were spotted onto sheep red blood agar plates and incubated at 28 °C. Zones of clearing were observed over a 15-h period.

environmental survival and/or acting as pathogens in a broad range of hosts, such as *My. marinum*, which has a larger genome than the pathogen *My. tuberculosis* (Stinear et al. 2008). Nevertheless, their weak virulence toward lepidopteran insects (Bisch et al. 2015) is not consistent with a broad host spectrum for *Xb* CS03, or for the *S. weiseri–Xb* CS03 pair. The *Xb* CS03 strain is also characterized by a large flexible genome, which may be highly dynamic, as already described for *X. nematophila* and *X. bovienii*, and for the phylogenetically close entomopathogenic bacterial genus *Photorhabdus* (Ogier et al. 2010; Murfin et al. 2015). The *Xb* CS03 genome contains more pseudogenes than the genome of *Xb* SS-2004, the only other available whole assembled genome for the *Xenorhabdus bovienii* species. This feature is surprising because *Xb* CS03 has a very large genome, whereas pseudogene proliferation is generally considered to be a hallmark of the initial stages of genomic reduction (Moran and Plague 2004). These genomic structure data reveal considerable differences from the genome of *X. poinarii* G6, another strain with attenuated virulence. *X. poinarii* G6 has a small genome with rare pseudogenes or insertion sequences. The observed genome reduction in this strain was mediated by the excision of genomic blocks from the flexible genome of the *X. poinarii* ancestor genome (Ogier et al. 2014). The pathologic and genomic properties of *Xp* G6 are common to all *X. poinarii* strains described to date (Akhurst 1986; Ogier et al. 2014). Furthermore, the association of *X. poinarii* with its nematode, *Steinernema glaseri*, is virulent (Akhurst 1986; Converse and Grewal 1998; Rosa et al. 2002; Ansari et al. 2003). In light of these data, we conclude that the evolutionary history of the genome of *Xb* CS03 differs radically from that of the species *X. poinarii*.

We investigated the content of the *Xb* CS03 genome further, by comparing genes encoded by *Xb* CS03 and *Xb* SS-2004. *Xb* CS03 was found to contain more genes potentially involved in interactions with other microbial competitors than *Xb* SS-2004: loci encoding restriction–modification systems, toxin–antitoxin systems, enzymes catalyzing the production of secondary metabolites, including numerous NRPS-PKS enzymes (Bode 2009; Singh et al. 2015). However, both strains have an *xnp1*-like locus encoding a tail-phage bacteriocin responsible for antimicrobial activity against bacteria phylogenetically close to *Xenorhabdus* (Morales-Soto et al, 2011). These genomic data were consistent with the pattern of

antimicrobial activity in *Xb* CS03, which targets phylogenetically distant bacterial species associated with the soil and insects rather than phylogenetically close bacteria. Competitor inhibition is an important strategy in the life cycle of *Steinernema–Xenorhabdus* pairs. If *Xenorhabdus* is able to persist for several weeks in the cadaver of the insect before reassociating with new generations of IJs and to protect the insect cadaver from competing soil microorganisms, it must produce numerous antimicrobial compounds (Nielsen-LeRoux et al. 2012). As *S. weiseri* emerges more slowly than other *Steinernema–Xenorhabdus* pairs (Bisch et al. 2015), competitor inhibition is probably crucial for the successful reassociation and emergence of the *S. weiseri-Xb* CS03 pair.

The second main difference between the *Xb* SS-2004 and *Xb* CS03 genomes is that the former contains a larger number of genes potentially involved in the exploitation of host resources. These genes include genes encoding virulence factors, as described in the virulence database (VFDB, http://www.mgc.ac.cn/VFs/): extracellular enzymes, secretion systems, toxins, adhesins, invasins, and iron-acquisition systems (Chen et al. 2005). In *Xb* CS03, several of these virulence loci (e.g., *tc* and *xaxAB*) have been pseudogenized by the insertion of transposons or prophages. This pattern of inactivation probably results from a relaxation of positive selection (Mira et al. 2001; Ochman and Davalos 2006).

According to the transmission-virulence trade-off hypothesis, the evolution of pathogens is driven by a positive link between virulence and the capacity to contaminate new hosts (Alizon and Michalakis 2015). A trade-off between efficient exploitation of the insect and inhibition of the competitive microorganisms in the insect cadaver may occur during the *Steinernema–Xenorhabdus* life cycle. Based on the differences between the genomes of *Xb* SS-2004 and *Xb* CS03 described here and previous descriptions of differences in their pathogenesis (Bisch et al. 2015), we suggest that this trade-off is heavily biased in *Xb* SS-2004 and *Xb* CS03. The fitness strategy of *Xb* SS-2004 seems to be based on efficient host exploitation, whereas that of *Xb* CS03 seems to rely more heavily on the inhibition of competitors.

What kind of evolutionary history could explain the specialization of *Xb* CS03 in competitor inhibition? We propose two nonmutually exclusive hypotheses. First, within the nematode–bacterium pair, the nematode partner *S. weiseri* 583 might be responsible for some of the virulence functions. For example, *Xb* CS03 is sensitive to antimicrobial peptides (Bisch et al. 2015). *Steinernema weiseri* may protect its bacterial symbiont by counteracting this component of the insect immune system, as has been demonstrated for other *Steinernema* species (Wang and Gaugler 1999; Brivio et al. 2006; Li et al. 2009; Liu et al. 2012). In this configuration, *Xb* CS03, the bacterial partner of the pair, would be able to specialize in the acquisition of genomic resources involved in microbial competition. Second, *Xb* CS03 may be evolving toward a saprophytic lifestyle. Indeed, *Steinernema–Xenorhabdus* pairs occasionally infest dead insects (San-Blas and Gowen 2008; Půza and Mrácek 2010). If the *S. weiseri* 583-*Xb* CS03 pair has developed the ability to multiply in insect cadavers, then outcompeting other microorganisms by producing a wide range of antimicrobial compounds may be crucial to its success. For both these hypotheses, an inability to kill the insect would be an evolutionary consequence. Indeed, maintaining a large set of synthetic enzymes involved in antimicrobial compound production is demanding in terms of energetic expenses, and probably leads to strong purifying selection on genes not essential for saprophytic microorganisms, such as the genes encoding virulence factors.

In conclusion, our analysis suggests that, within the *Xb* species, strains have had very different evolutionary genomic histories. To date, only a few genomic scenarios have been described for bacteria alternating between different ecological niches exerting opposite evolutionary pressures. We suggest that this configuration generates a multiplicity of genomic patterns and, consequently, a multiplicity of strategies for interaction with hosts.

Supplementary Material

Supplementary tables S1–S9 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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