JOURNAL OF BACTERIOLOGY, Aug. 2003, p. 4648–4656 Vol. 185, No. 15
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.15.4648–4656.2003

Using a DNA Microarray To Investigate the Distribution of Insect Virulence Factors in Strains of *Photorhabdus* Bacteria

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Received 19 February 2003/Accepted 5 May 2003

***Photorhabdus* is an insect-pathogenic bacterium in which oral toxicity to insects is found in two distinct taxonomic groups. Using a DNA microarray and comparative genomics, we show that oral toxicity is associated with toxin complex genes *tcaABC* and that this locus can be mobilized or deleted within different strains.**

DNA microarrays have been used to look at the relatedness of strains of bacteria pathogenic to humans (10, 15). Arrays from one strain of bacteria are useful in identifying genes absent, or highly divergent, in unsequenced test strains (15). The data obtained are restricted to the genes present in the reference strain; however, comparisons of the similarity of unknown genomes to the reference strain can be made (6). Further, the presence, absence, or divergence of specific genes can be correlated with phenotypes either shared by, or lacking in, one or another of the strains. Although arrays have been used to study obligate symbionts of insects (1, 2), here we study an insect pathogen.

Photorhabdus bacteria are insect pathogens belonging to the family *Enterobacteriaceae* (11). The bacteria reside in the guts of nematodes that invade insects and are released directly into the open blood system of the insect. They replicate within and kill the insect host (7) by using insecticidal toxins such as the toxin complexes (Tc's) (20) and the makes caterpillars floppy (Mcf) (5) toxin. Sequencing of different *Photorhabdus* genomes has confirmed that they contain a wide range of putative virulence factors, including toxins, exoenzymes, and hemolysins (7). We are interested in examining the relatedness of the genomes of orally toxic and nontoxic strains, with specific reference to the genes involved in oral toxicity, the tc genes. The tc genes are members of a large gene family and are found in multiple copies in individual *Photorhabdus* genomes (20). Four different family members, *tca*, *tcb*, *tcc*, and *tcd*, have been described on the basis of a description of the first four loci cloned from *Photorhabdus luminescens* strain W14 (3). Genetic knockout of either *tca* or *tcd* greatly reduces the oral toxicity of the resulting supernatant in the associated W14 mutants (3). Here we determine the phylogeny of orally toxic and nontoxic strains via analysis of their 16S DNA sequences (9) in order to test the simplest hypothesis that oral toxicity is found in a single related group of strains. We also hybridize genomic DNA from each strain to a microarray carrying 96 putative virulence factor genes from the orally toxic *Photorhabdus* strain W14 in order to investigate the minimal subset of tc genes required for oral toxicity.

Oral toxicity is found in two separate groups. The oral toxicity of the strain supernatants (Table 1) was assessed (19), and the oral toxicity of the washed bacterial cells was examined alongside that of the supernatant itself. Supernatants causing a 50% or greater reduction in growth at 7 days, relative to that in broth-only controls (data not shown), were classed as orally toxic. 16S ribosomal DNA PCR was performed (9), products were sequenced, and a well-supported neighbor-joining tree was constructed (Fig. 1). This phylogeny is very similar to that determined by others using a neighbor-joining method (17). The six orally toxic strains fall into two distinct groups. The first (IS5, EG2, IND, and W14 itself) lies within the previously recognized *Photorhabdus luminescens* subsp. *akhurstii* subspecies. The second (Hb1 and Hm1) represents a different subgroup, *Photorhabdus luminescens* subsp. *luminescens*. Each of the four nontoxic strains occupies its own independent taxonomic group. This analysis also shows that some other strains do not fit readily within the existing classification (labeled "*Photorhabdus* sp." in Fig. 1); however, their likely reclassification is not discussed here.

Conserved and variable genes in the array analysis. We cohybridized labeled genomic DNA from the nine *Photorhabdus* test strains with labeled genomic DNA from the control strain, W14, against a 96-gene microarray based on likely virulence factors from the orally toxic strain W14. The genes were

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TABLE 1. *Photorhabdus* and *Xenorhabdus* strains, their geographic origins, and their nematode hosts[a]

Strain	Origin	Source	Host
Photorhabdus			
Hb	Victoria, Australia	R. Akhurst	*Heterorhabditis bacteriophora*
Hm	Georgia	K. Nealson	*Heterorhabditis* sp.
W14	Florida	D. Bowen	*Heterorhbditis* sp.
IND	Antibes	A. Fodor	*Heterorhabditis indica*
EG2	Egypt	A. Fodor	*Heterorhabditis indica*
IS5	Israel	A. Fodor	*Heterorhabditis indica*
TT01	Trinidad	D. Clarke	*Heterorhabditis bacteriophora* HP88
Brecon	South Australia	A. Fodor	*Heterorhabditis bacteriophora*
HP88	Utah	A. Fodor	*Heterorhabditis bacteriophora* HP88
Az36	Azores	N. Simoes	*Heterorhabditis bacteriophora*
ARG	Argentina	A. Fodor	*Heterorhabditis bacteriophora*
K122	Ireland	D. Clarke	*Heterorhabditis downesi*
HSH2	Germany	R. Ehlers	*Heterorhabditis megidis*
HL81	The Netherlands	F. Galle	*Heterorhabditis megidis* (NWE)
H4	Hungary	A. Fodor	*Heterorhabditis megidis*
Meg1	Ohio	A. Fodor	*Heterorhabditis megidis*
OH1	Ohio	A. Fodor	*Heterorhabditis megidis*
NC19	North Carolina	R. Akhurst	*Heterorhbditis bacteriophora*
Heliothidis	United States	A. Fodor	*Heterorhabditis bacteriophora*
Wx6	Wisconsin	K. H. Nealson	*Heterorhabditis* sp.
Wx8	Wisconsin	K. H. Nealson	*Heterorhabditis* sp.
P7	Canada	L. Gerritsen	*Heterorhabditis megidis*
Wx9	Wisconsin	K. H. Nealson	*Heterorhabditis* sp.
Wx10	Wisconsin	K. H. Nealson	*Heterorhabditis* sp.
Wx11	Wisconsin	K. H. Nealson	*Heterorhabditis* sp.
Wx12	Wisconsin	K. H. Nealson	*Heterorhabditis* sp.
Wx9Hyper	Wisconsin	K. H. Nealson	*Heterorhabditis* sp.
KOH	Hungary	A. Fodor	*Heterorhabditis bacteriophora*
Az29	Azores	N. Simoes	*Heterorhabditis bacteriophora*
MOL	Moldavia	L. Gerritsen	*Heterorhabditis bacteriophora*
JUN	The Netherlands	L. Gerritsen	*Heterorhabditis megidis*
HIT	Hungary	C. Griffins	*Heterorhabditis* "Irish" type
ATCC43949 (ASY)	CDC,[b] Atlanta, Ga.	J. Farmer	Human blood
Xenorhabdus			
DSM4766	Tasmania, Australia	R. Akhurst	*Steinernema feltiae*
DSM3370 (X3)	Georgia	R. Akhurst	*Steinernema carpocapsae*
DSM4764	Queensland, Australia	R. Akhurst	*Steinernema* sp.

[a] Accession numbers for the 16S DNA sequences are AY278640 to AY278675.
[b] CDC, Centers for Disease Control and Prevention.

divided into those that were "conserved" (values between 2 and 0.5 for all strains) and those that were "variable" (values under 0.5 for some strains) between strains (Fig. 2). Noting the limitations of fixed cutoff points for presence-absence prediction (14) we confirmed the predicted results of the array via sequence data. Only one locus, an erythrocyte lysis protein 2-like gene, appears to be unique to W14, whereas all the other genes occur in one or more of the *Photorhabdus* strains studied. Thus, the *lux* genes, producing light, a phenotype common to all *Photorhabdus* bacteria (12), are found in all strains. A series of putative virulence factors also appear conserved in most or all strains, including the toxin-encoding gene *mcf1* (5), *rtxA1*- and *rtxA2*-like genes, the operon encoding the *prtA* protease, and an attachment and invasion locus (*ail*) homolog (7). The type III secretion system (18), often associated with virulence in other gram-negative bacteria (13), is also present in all strains. Other conserved genes include those encoding catalase, chitinase, ferrochelatase, and flagellae.

Variable genes include those encoding bacteriocins, toxins and hemolysins, insertion elements, and pili, as well as genes involved in iron acquisition. The lumicins are novel bacteriocins described from *Photorhabdus* strain W14 (16). The lumicin loci consist of killer protein genes followed by multiple mixed-type immunity genes, whose role is to counter the effect of the killer protein on the host cell. Within W14 there are four predicted loci, *lum1*, *lum2*, *lum3*, and *lum4* (16), of which the array predicts *lum1* and *lum2* to be variable and *lum3* to be conserved between strains (Fig. 2). Comparison of the W14 and TT01 *lum1* sequences (Fig. 3) shows that while the *lum1B* E4-type immunity protein gene is conserved between the two strains, the killer protein itself (*lum1A*) and the *lum1C* S3-type immunity protein genes are divergent. Divergence in killer protein and immunity protein sequences between strains is expected in the likely presence of intense interstrain competition within the insect host (16).

Some toxin and hemolysin-hemagglutinin genes are also

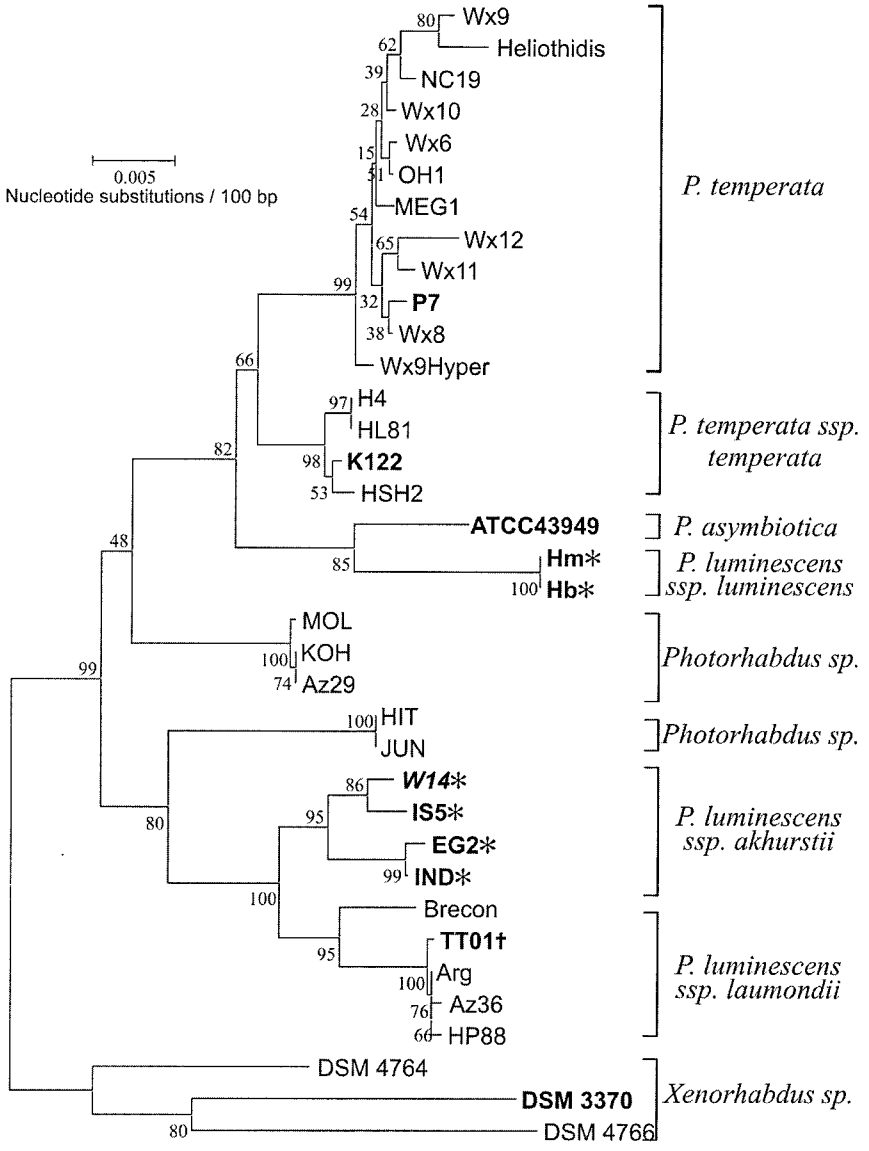
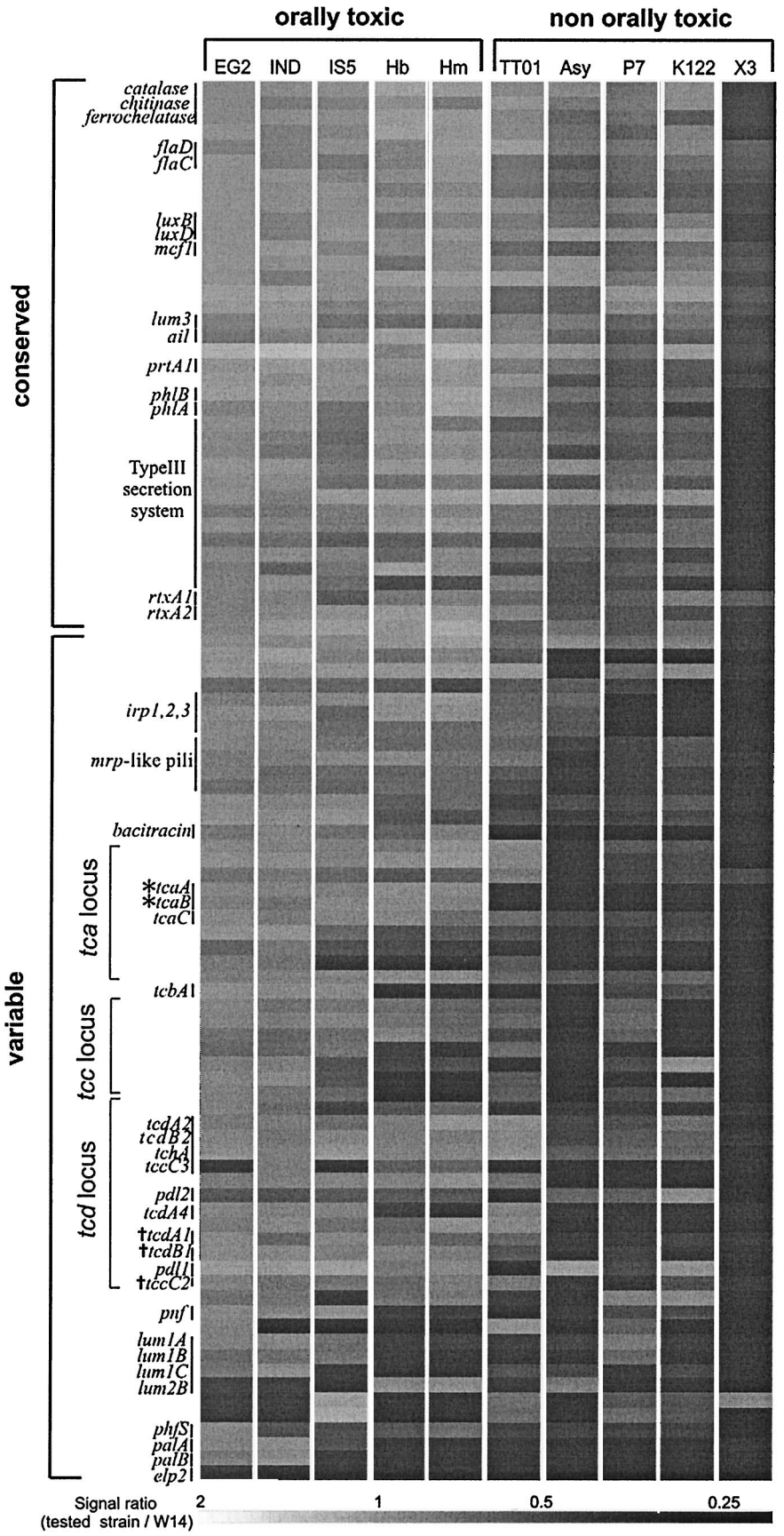


FIG. 1. Phylogeny of *Photorhabdus* strains (Table 1) highlighting the two groups showing orally toxic culture supernatants. Note that oral toxicity is found in two well-supported subspecies, *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp *luminescens* (termed groups 1 and 2). The tree was constructed by using Kimura's two-parameter model of distance estimation and running 1,000 bootstrap replicates with the MEGA program (Molecular Evolutionary Genetics Analysis, version 2.1; Arizona State University).

variable between strains. These include the *pnf* gene, encoding a *Photorhabdus* necrotizing factor, which is found within a recently acquired region of the W14 genome (18) that is absent from TT01, and the *palA* and *palB* genes, encoding a hemolysin-hemagglutinin and its export activator (18). In W14 (Fig. 3), *palBA* is immediately downstream of the *mcf1* toxin gene and adjacent to a Phe tRNA (18), whereas in TT01 (Fig. 3), *palBA* is deleted from the equivalent location, as predicted. In

FIG. 2. Hybridization ratios for the 96 genes in the W14 array hybridized against the nine *Photorhabdus* strains and one *Xenorhabdus* strain. *Photorhabdus* strains are arranged into those showing orally toxic supernatants and those showing nontoxic supernatants. The 96 W14 genes are arranged into groups predicted to be present (conserved) in most *Photorhabdus* strains or absent in some strains (variable). Specific groups of genes discussed in the text are labeled. A hybridization ratio of >2 predicts more than one copy of a gene; 0.5 to 2 predicts the presence of a single copy of the gene or a homolog; and <0.5 predicts the absence, or significant sequence divergence, of the gene. A table of gene names, putative functions, and the sequences of the arrayed PCR products is posted at http://staff.bath.ac.uk/bssrffc/downloads.html as supplementary information.


orally toxic
non orally toxic
EG2
IND
IS5
Hb
Hm
TT01
Asy
P7
K122
X3
conserved
variable
catalase
chitinase
ferrochelatase
flaD
flaC
luxB
luxD
mcfl
lum3
ail
prtA1
phlB
phlA
TypeIII secretion system
rtxA1
rtxA2
irp1,2,3
mrp-like pili
bacitracin
tca locus
*tcaA
*tcaB
tcaC
tcc locus
tcbA
tcd locus
tcdA2
tcdB2
tchA
tccC3
pdl2
tcdA4
†tcdA1
†tcdB1
pdl1
†tccC2
pnf
lum1A
lum1B
lum1C
lum2B
phfS
palA
palB
elp2
Signal ratio (tested strain / W14)
2
1
0.5
0.25

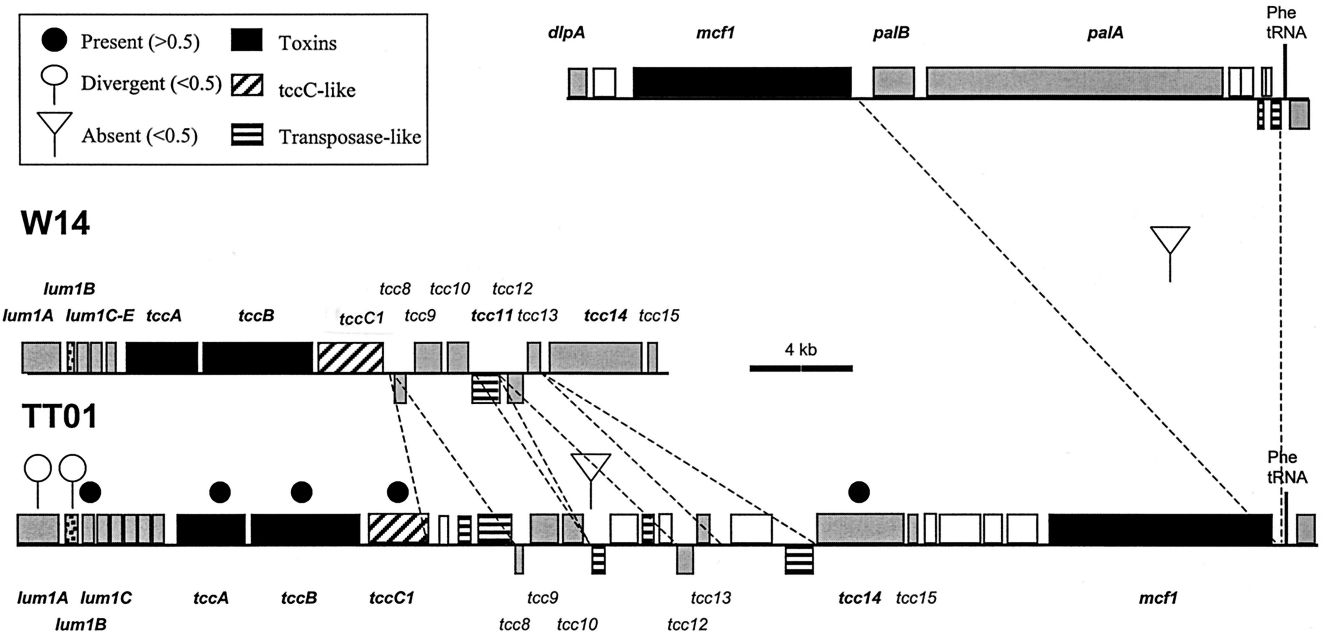


FIG. 3. Comparative genomic organization of the region encompassing the *lum1* and *tcc* loci in the different *Photorhabdus* strains W14 and TT01. Solid circles, open circles, and inverted triangles above the open reading frames indicate the predicted presence, divergence, and absence of genes, respectively, according to hybridization to the microarray. Other symbols and dashed lines indicate the locations of specific deletion or insertion events within the two genomes (see the key). Note that all the genes of the *tcc* locus are present in both strains and are also linked to the *mcf* locus in TT01. Sequences are from GenBank accession no. AF346499 and AF503504 and from World Intellectual Property 02/094867.

contrast, a second two-component hemolysin locus, *phlAB* (4), is predicted to be similar in all strains and indeed to be potentially duplicated in strain Hm. The *mrp*-like pili and the PhfS fimbrial major subunit gene show a gradual change in hybridization ratio across strains, suggesting that they are present in all strains but may diverge in sequence (18). Finally, the *Photorhabdus irp*-like genes (8), encoding yersiniabactin biosynthetic protein homologs, predicted to synthesize an iron-scavenging siderophore, are present in all but strains K122 and P7.

Oral toxicity and the tc genes. The tc genes are variable genes (Fig. 2) whose distribution is strikingly split between orally toxic and nontoxic strains (Fig. 4). Orally toxic strains carry all three genes in the *tca* operon (*tcaA*, *tcaB*, and *tcaC*), whereas those lacking toxicity lack *tcaA* and *tcaB*. The genes of the *tca* operon are the only genes to show a perfect correlation with oral toxicity; genes from the *tcb*, *tcc*, and *tcd* operons are all variable across orally toxic strains.

For the *tca* locus (Fig. 5), a comparison of W14 and TT01 shows two important findings. First, the W14 *tcaABC* operon is absent from the equivalent location in TT01. Second, a *tca*-like locus is present elsewhere in TT01 but lacks most of *tcaA* and *tcaB*, which have been deleted, and retains only a *tcaC1*-like gene. These observations confirm that the presence of *tcaAB* is necessary for the oral toxicity of the bacterial supernatant. Second, the fact that *tca*-like loci can be found at different locations in different genomes supports the concept that the *tca* locus is mobile, as suggested by the presence of either a transposase (in W14) or an integration protein (in TT01) adjacent to the *tca*-like locus (Fig. 5).

For the *tcb* locus (Fig. 6), the suggestion that *tcbA* is lacking from TT01 is again confirmed by analysis of the genomic sequence: *tcbA* is deleted from the equivalent location in TT01, and a transposase is left in its place. Again comparing W14 and TT01, the array suggests that the *tcc* locus should be completely conserved, as supported by an examination of the genomic sequence (Fig. 3). Finally, for *tcd*, the genomic sequence (Fig. 7) confirms that all the tc genes of the island are present in both W14 and TT01, including both *lysR*-like regulators. Further, the predicted loss of *tcdA4* in group 2 orally toxic strains is confirmed by an examination of this locus in the Hb strain. The array even predicts the loss of *pdl1* and *pdl2* from within the *tcd* island of TT01, which, again, is confirmed by the sequence (Fig. 7). The localized deletion of *tcdA4* and the apparent rearrangements mediated by *tccC*-like genes support the hypothesis that the *tcd* genes are encompassed in an unstable pathogenicity island. However, the consistent maintenance of four genetic elements within this island (*tcdA2*, *tcdB2*, a *gp13*-like holin, and the conserved region of *tccC3*), and the conserved organization of similar *tcd*-like genes in other bacteria (such as *sepA*, *sepB*, *orf4*, and *sepC* in *Serratia* spp.), suggests that these genes form an invariant, but not orally toxic, core.

Our previous analysis of strain W14 shows that either *tca* or *tcd* can independently contribute to oral toxicity. Thus, plasmid clones of either *tca* or *tcd* from W14 confer recombinant oral toxicity when expressed in other, non-orally toxic *Photorhabdus* strains, such as K122. These data are consistent with the present observation that the presence or absence of

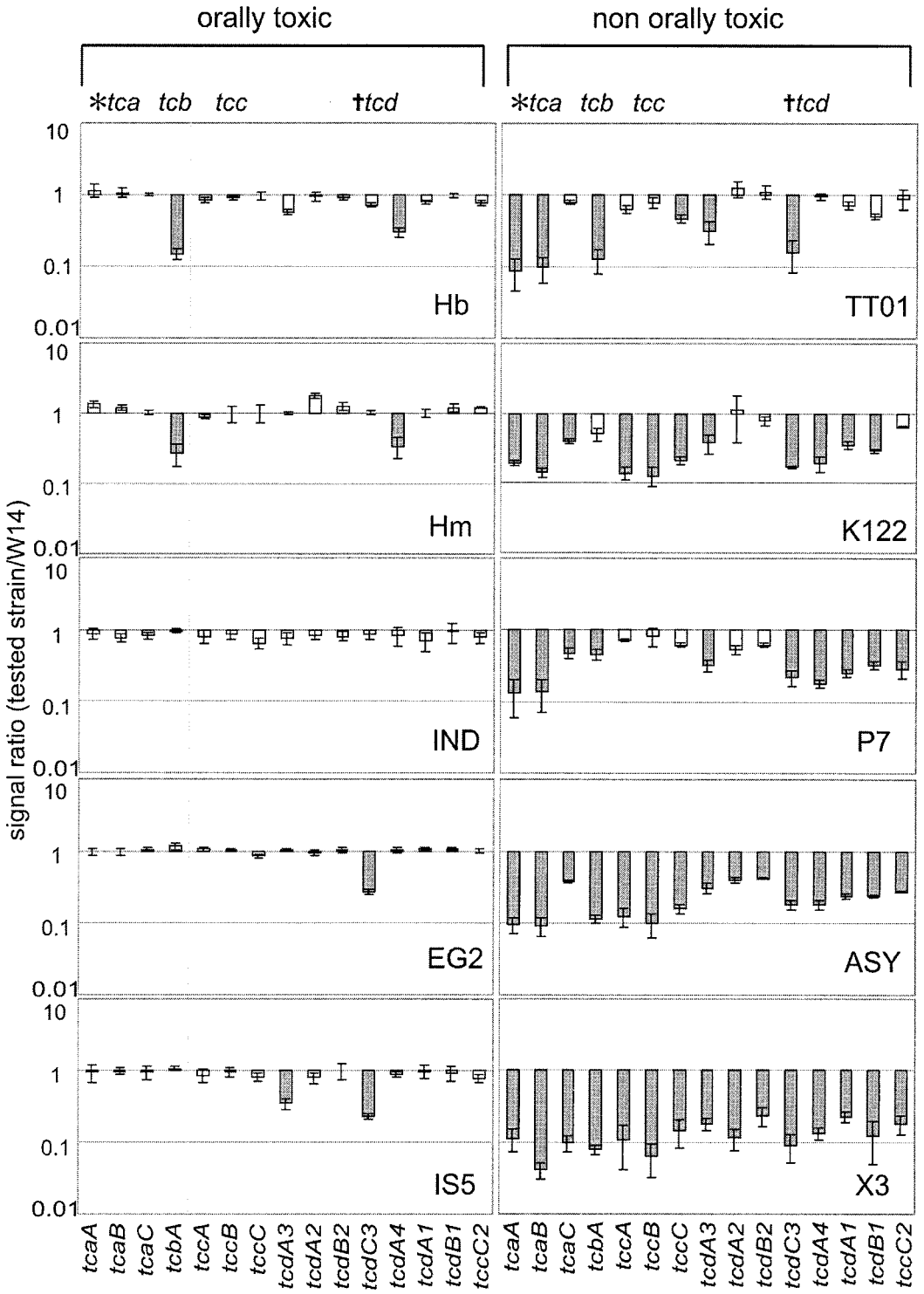


FIG. 4. Hybridization ratios of genes in the *tca*, *tcb*, *tcc*, and *tcd* loci for strains with orally toxic (left) or nontoxic (right) supernatants. Values close to 1.0 predict the conservation of a gene between strains (open bars), and values of <0.5 predict its absence or sequence divergence (shaded bars). Note that *tcaA* and *tcaB* are the only genes perfectly correlated with the presence of oral toxicity.

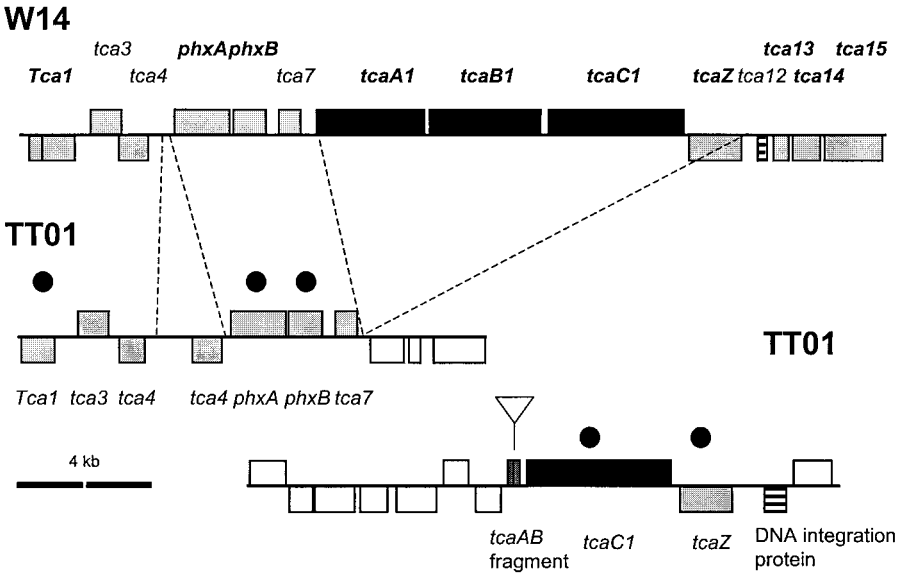


FIG. 5. Comparative genomic organization of the *tca* locus in the different *Photorhabdus* strains W14 and TT01. Note that the entire W14 *tcaABC* operon is deleted from the equivalent location in the TT01 genome but that a *tcaC*-like gene is found at a different location in the TT01 genome, next to a copy of *tcaAB* with an internal deletion. This suggests that the *tca* operon is mobile and can occupy different genomic locations in different strains. See the key in Fig. 3. Sequences are from GenBank accession no. AF346497 and World Intellectual Property 02/094867.

tcaAB is perfectly correlated with the toxicity of the supernatant. However, all the tc genes within the *tcd* island are also present in some strains, such as TT01 (Fig. 7), which lack orally toxic supernatants. To investigate the apparent lack of oral toxicity associated with *tcd* in TT01, we examined the toxicity of the bacterial cells independently of their supernatant and found that toxicity is associated with the cells rather than the supernatant (Fig. 8). Confirmation that this novel cell-associated toxicity is *tcd* related now requires knockout or heterologous expression of this locus. In conclusion, even a very limited microarray can provide a powerful predictive tool for correlating observed phenotypes with bacterial genotypes. Similar microarrays may therefore be useful in investigating other *Photorhabdus* phenotypes involved either in insect pathogenicity or in symbiosis with the nematode hosts of these bacteria.

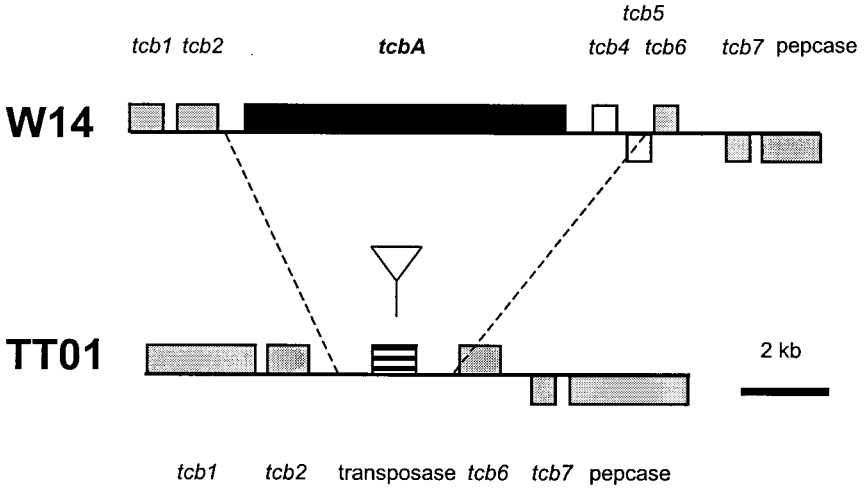


FIG. 6. Comparative genomic organization of the *tcb* locus in the different *Photorhabdus* strains W14 and TT01. Note that the *tcbA* gene is deleted from the equivalent location in the genome of TT01 and is replaced by a transposase gene, which is potentially implicated in its excision. See the key in Fig. 3. Sequences are from GenBank accession no. AF346498 and World Intellectual Property 02/094867.

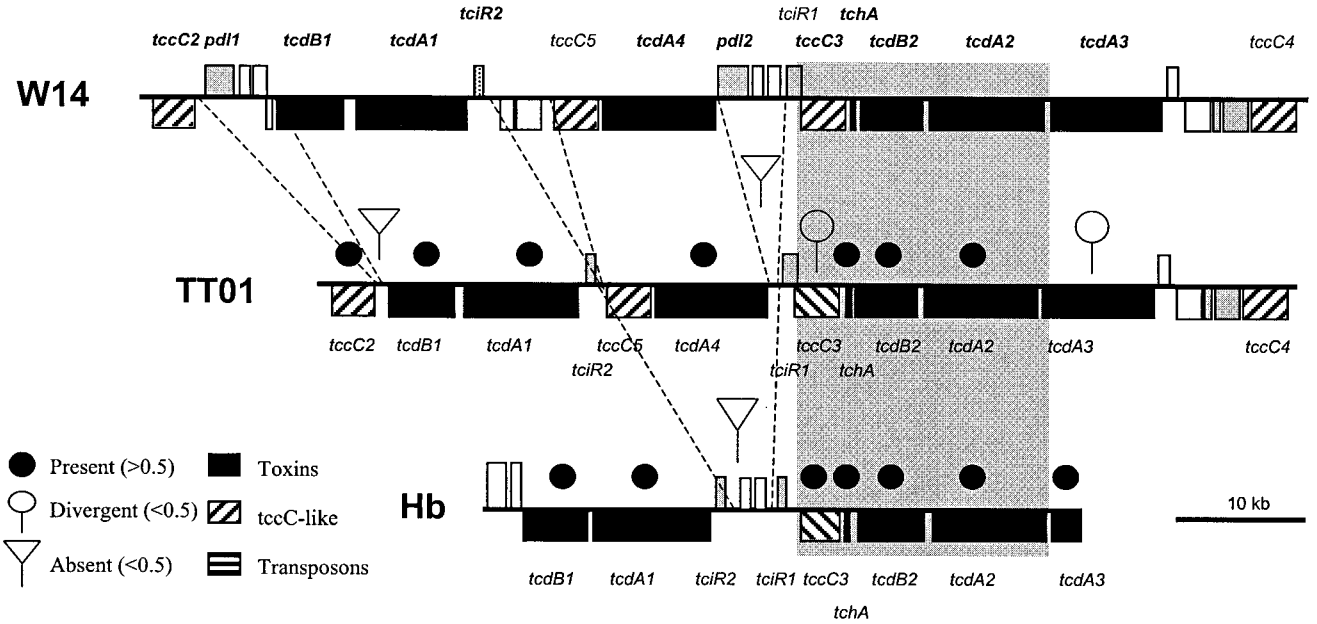


FIG. 7. Comparative genomic organization of the *tcd* pathogenicity island in the different *Photorhabdus* strains W14, TT01, and Hb. Note that despite the presence of local deletions and insertions, W14 and TT01 share all of the tc genes within the *tcd* locus, whereas strain Hb lacks a *tcdA4*-like gene, as predicted by the array. The positions of other genes discussed in the text are indicated. Sequences are from GenBank accession no. AY144119 and World Intellectual Property 02/094867 and 99/42589.

This work was supported by grants to R. H. f.-C. from the Exploiting Genomics Initiative of the Biotechnology and Biological Sciences Research Council of the United Kingdom and by a Marie Curie Ph.D. training grant to J.M. We also acknowledge The Wellcome Trust for funding the Bacterial Microarray Group at St George's Hospital Medical School under its Functional Genomics Resources Initiative.

We thank the laboratories of David Clarke and Stuart Reynolds for useful discussions.

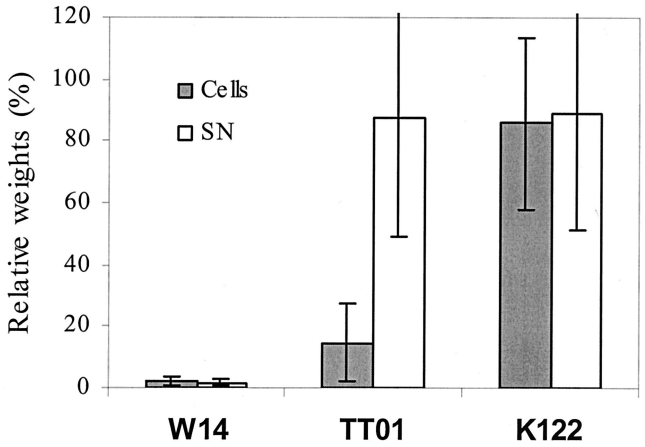


FIG. 8. Comparison of the oral toxicity associated with the bacterial supernatant (SN) and that associated with washed bacterial cells, expressed as percentages of weights of untreated controls. Note that cells, but not supernatants, from strain TT01 show oral toxicity, correlating with the presence of genes in the *tcd* locus. Only strains carrying *tcaAB* (for example, W14) show orally toxic supernatants.

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