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TITLE PAGE

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PRINCIPAL INVESTIGATOR

Robert Charles Wood, Ph. D.

INSTITUTION

George Washington University School of Medicine, Washington 5, D. C.

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ABSTRACT

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Alterations were observed in the virulence of Shigella flexneri 2a for pre-
treated guinea pigs following hybridization by Escherichia coli B-12 Mr strains.
Genetic studies indicated extensive chromosomal homology between these two species.
However, recombination frequencies were lower than those obtained in comparable
E. coli X B-12 crosses, and extensive transfer of the Escherichia genome was
detected only occasionally. Virulence tests on Shigella hybrids carrying over-
lapping segments of the Escherichia chromosome revealed only one region, located
between the $r$ and $x$ genes, which was essential for virulence. Hybrids
carrying the $x$ region as a predominant congener displayed
intermediate virulence; haploid segregants which lost this congener were fully
virulent, while one haploid which had incorporated it proved to be avirulent.

Preliminary tests on a mouse-virulent Salmonella typhi murium hybridized by an
avirulent Salmonella abortus donor indicated that, in this species also, virulence
may be lost on substitution of the \textit{xyl}^+ - \textit{rha}^+ region. \textit{Salmonella typhosa} strain 64365*, hybridized by mating with an \textit{E. coli} Mfr strain, was found to exhibit enhanced recipient ability on remating with \textit{E. coli} donors whose lead chromosomal region matched the genetic segment integrated by the hybrid.
1. Alterations in the virulence of *Escherichia-Shigella* hybrid for the guinea pig.

1. During the past year this investigation has focused on the genetic basis of virulence in enteric bacteria. One important finding has been the loss of guinea pig virulence in the 2a strain of *Shigella flexneri* after specific substitution of *Escherichia coli* genetic material, as a result of mating with *E. coli* K-12 Bfr strains.

2. Genetic recombination between Bfr strains of *Escherichia coli* K-12 and members of the *Shigella* group was initially demonstrated by Luria and Burrows (1937). Extensive, though incomplete, genetic homology was found to exist between these two genera. Formal et al. (1956, 1959) demonstrated a fatal enteric infection in either starved or carbon tetrachloride pretreated guinea pigs challenged orally with *E. flexneri* 2a. This infection produced lesions in both the large and small bowel which resembled those seen in human cases of shigellosis. The availability of both a genetic transfer system and an infection model thus enabled examination of alterations in the virulence of *E. flexneri* 2a for pretreated guinea pigs, following conjugation with *E. coli* K-12.

3. Three *E. coli* K-12 Bfr strains were employed in the genetic crosses: W395, which transfers its chromosomal markers in the order origin, lactose utilisation (leu*'), arabinose utilisation (ara*'), rhamnose utilisation (rha*'), indol production (ind*'), xylose utilisation (xyl*'), maltose utilisation (mal*'), fructose utilisation (fuc*'), nicotinic acid synthesis (nia*'), galactose utilisation (gal*'); MD 313 (origin, xyl*', xyl*'; mal*';... rha*'); 1362
(origin, *mal*, *xyl*, *ile*, ....*fuc*). Matings between these *E. coli* donors and the 2a *E. coli* strain indicated that a gross homology exists between the chromosomes of these two species. However, recombination frequencies were lower than those obtained in comparable *E. coli* X *E. coli* crosses (Table 1). Further, the predominant *Shigella* hybrid class acquired only the selected genetic marker from the *E. coli* parent; extensive transfer of the *Escherichia* genome was detected only occasionally (Table 2).

4. A definite progressive relation between dosage and response is not always apparent in the oral administration of *Shigella* to starved or carbon tetrachloride-treated guinea pigs (Formal et al., 1958, 1959). Thus it was not possible to employ the DSO dose as an estimate of virulence. However, it was observed that a dose of 5 X 10⁷ to 1 X 10⁸ *Shigella* organisms administered orally, under our experimental conditions, consistently killed a large proportion of animals. Assessment of virulence of parental and hybrid strains, therefore, was dependent primarily on an "all or none" type of response.

5. By testing *Shigella* hybrids carrying overlapping segments of the *Escherichia* genome, it was hoped that the minimum number of chromosomal regions responsible for the virulence of *Shigella* for the pretreated guinea pig might be identified. Our virulence data is summarized in Table 3. Hybrids having received *lac*⁺, *lac*⁻, *xyl*⁺, *xyl*⁻, and *mal*⁺ from the *E. coli* parent were as virulent as the *Shigella* parental strain. Those hybrids which carried the *mal*⁺ were uniformly avirulent, while *xyl*⁺ and *xyl*⁻ *mal*⁺ hybrids were either completely virulent or avirulent in about equal proportions. Although one *fuc*⁺ hybrid was virulent, the remaining *fuc*⁺ hybrids tested, as well as all of the *mal*⁺, *fuc*⁺, *nic*⁺, and *fuc*⁻ *nic*⁻ *lac*⁺ were of intermediate virulence, i.e., they consistently killed a low but significant proportion of animals. In addition, hybrids which had received the *P.3* antigen from *E. coli* also displayed this intermediate virulence.
6. Examination of hybrids possessing the characteristics of segregating partial diploids strongly indicated that the observed alterations in virulence were due to the transferred Escherichia material. Several rha<sup>+</sup> - ind<sup>+</sup> - xyl<sup>+</sup> - mal<sup>+</sup> hybrids, in spite of repeated purification, segregated clones (about 1 in 10<sup>4</sup> cells) which exhibited the parental Shigella phenotype. When tested in the guinea pig (Table 4), these partial diploids were found to be of intermediate virulence, while their haploid segregants, which had completely lost the injected Escherichia genes, were indistinguishable from the parental Shigella strain in their virulence. On one occasion a haploid segregant was isolated in which the previously exogenetic fragment had become integrated. This segregant proved to be avirulent.

II. Alterations in the virulence of Salmonella - Salmonella hybrids for the mouse.

1. The indication of a genetic locus, or loci, controlling the virulence of Shigella for the pretreated guinea pig has led us to investigate the possible existence of a similar factor, or factors, in Salmonella. Hybridization of Salmonella by E. coli was initially reported by Baron, Carey and Spilman (1958). Subsequently, it has been found possible to produce Salmonella possessing donor ability, either by hybridization for a terminal E. coli Hfr marker (which often results in integration of the sex factor, F), or by infection with F, followed by selection for Hfr mutants. The use of such donors in crosses with Salmonella typhimurium, a natural pathogen for the mouse, presents an excellent system for the genetic study of virulence.
2. In preliminary experiments, S. typhimurium strain C-5, genetically marked and virulent for the mouse, was mated with an avirulent S. sherry donor strain, and several hybrid classes from this mating were tested for mouse virulence. The initial findings indicate that virulence may be lost in the hybrids when substitution occurs in the \( xyl^+ - rha^+ \) region. The correspondence of this preliminary data with that obtained from the experiments with the Shigella hybrids is most encouraging. However, further studies will be necessary to confirm these findings, and to establish whether other chromosomal regions might also be involved in the virulence of S. typhimurium for the mouse.

III. Behavior of E. coli - S. typhosa hybrids on remating with E. coli Hfr strains.

1. From the outset of this investigation our hope has been to transfer the property of virulence from a virulent genetic donor to an avirulent genetic recipient. As yet, this has not been accomplished, owing to the difficulty of obtaining virulent donor strains. Salmonella, either virulent or avirulent, generally are poor recipients in genetic crosses with E. coli K-12 donors. Transfer of Hfr donor ability to virulent Salmonella strains in these cases is very hard to achieve. However, our studies on the behavior of S. typhosa hybrids produced by mating with E. coli have pointed out a method by which the genetic recipient ability of Salmonella may be increased.

2. It has been previously observed that S. typhimurium hybrids from genetic crosses with E. coli Hfr strains exhibit increased fertility when remated with the E. coli parent. This has been shown to represent selection of pre-existing, high frequency recipients from an otherwise sterile population by the first round of mating. However, upon examination of this phenomenon with S. typhosa as the genetic recipient, we discovered that, in this species, selec-
tion of high frequency recipients was not involved. Our studies showed, in fact, that the increased recipient ability of the *S. typhosa* hybrids was due to the presence of integrated *E. coli* genetic material.

3. *S. typhosa* strain 643WSR was mated with the *E. coli* Hfr W1895 (origin, lac+, ara+, ... gal+) with selection for the lead marker lac+. Lactose positive *S. typhosa* hybrids, when remated with W1895 for the more distal marker ara+, showed frequency increases of 200 times the normal frequency for transfer of this gene to previously unmated *S. typhosa*. It was noted that many lac+ *S. typhosa* hybrids were heterogonotes which continually segregated lactose negative (lac-') clones. When a number of these lac- segregants, which had lost the *E. coli* genetic material, were remated with the *E. coli* parent, none showed any increase in recipient ability. This provided the initial evidence that the fertility increases of the hybrids occurred as a consequence of the transferred *E. coli* genetic material.

4. *E. coli* Hfr strains W1895 and Hayes (transferring origin, ara+, lac+, ... rho+) were crossed with *S. typhosa* 643 WS*, with selection for the single markers lac+ and ara+. Four classes of 643WSR hybrids were obtained which were labelled as follows: those receiving lac+ alone or ara+ alone from W1895 were designated W5R lac+(95) and W5R ara+(95), respectively; those receiving these individual markers from Hayes were labelled W5R lac+(H) and W5R ara+(H). These four hybrid classes were then remated with each of the two *E. coli* Hfr strains in a series of eight reciprocal genetic crosses. These crosses, shown in Table 5, demonstrated that each hybrid class was capable of exhibiting significant increases in recombination frequency wherever the leading chromosomal region of
the _E. coli_ Hfr used for remating matched the _E. coli_ genetic segment previously integrated by the hybrid. When the alternate Hfr (which did not inject its chromosome into the region of artificial genetic homology) was employed, no increase in recombination frequency was observed. An exception was noted in the Hayes X WB^R lec^+(H) matings (Table 5, cross 6), where a 13-fold increase occurred when the _E. coli_ segment integrated by the hybrid does not appear to match the lead chromosomal region of Hayes. Probably, these hybrids have integrated _E. coli_ material nearer to the Hayes lead region than the absence of the ara^+ marker would indicate.

5. Thus, it is possible to increase significantly the recipient ability of _S. typhosa_ in crosses with _E. coli_ Hfr strains by transfer and integration of strategically located _E. coli_ chromosomal segments. The studies of Zinder (1960) and Falkow, Rowd and Baron (1962) have shown that the homology which exists between _Escherichia_ and _Salmonella_ is incomplete. Presumably, then, prior establishment in _S. typhosa_ of an _Escherichia_ genetic segment homologous with the leading chromosomal section of the _E. coli_ donor facilitates early genetic pairing and integration of the transferred material. Removal of the initial barrier to integration of lead Hfr markers would increase the chances of integration of more distal markers, thus accounting for the observed increase in recombination frequencies.

**Summary**

1. Three _E. coli_ K-12 Hfr donors were employed in genetic crosses with the 2a strain of _Shigella flexneri_. The results of these matings confirmed the existence of a gross homology between the chromosomes of these two organisms.
However, recombination frequencies were lower than those obtained in comparable
*E. coli* × *E. coli* crosses, and extensive transfer of the *Escherichia* genome was
detected only occasionally.

2. *Shigella* hybrids carrying overlapping segments of the *Escherichia*
genome were compared with the parental *Shigella* strain with regard to their
virulence for starved or carbon tetrachloride treated guinea pigs. Hybrids
which received *lac*⁺, *lac*⁻ *ara*⁺, *rha*⁺, and *mal*⁺ from the *E. coli* parent were
as virulent as the parent strain. Hybrids carrying *rha*⁺ *ind*⁺ were uniformly
avirulent, while *xyl*⁺ and *xyl*⁺ *mal*⁺ hybrids were either completely virulent
or avirulent in about equal proportions. With one exception, *fuc*⁺, *nic*⁺,
*fuc*⁺ *nic*⁺, and *fuc*⁺ *nic*⁺ *lac*⁺ hybrids, as well as those receiving the
pili antigen from *E. coli*, were of intermediate virulence.

3. *Shigella* *rha*⁺ *ind*⁺ *xyl*⁺ *mal*⁺ hybrids, possessing the character-
istics of segregating heterogenotes, were found to be of intermediate virulence,
while their haploid segregants, which had lost the *E. coli* genetic material,
were fully virulent. One haploid segregant, which had integrated the pre-
viously exogenetic *E. coli* fragment, proved to be avirulent.

4. In preliminary experiments, a virulent *Salmonella typhimurium* strain,
C-5, was mated with an avirulent *Salmonella* *abony* donor strain. Testing of
several hybrid classes produced by this mating indicated that mouse virulence
may be lost in the hybrids when substitution occurs in the *xyl*⁺ *rha*⁺ region.

5. Lactose positive *Salmonella typhosa* hybrids produced by mating with
*E. coli* Hfr W1895 showed significant increases in frequency when remated with
this Hfr. However, lactose negative segregants from lac⁺ S. typhosa hetero-
genotes displayed no fertility increase, indicating that the integrated Escher-
ichia genetic material was responsible for the enhanced recipient ability.

6. E. coli Hfr strains W1895 and Hayes were mated with S. typhosa 643RS⁺
with single marker selection for lac⁺ and ara⁺. Four hybrid classes were ob-
tained, each possessing a single marker derived from one of the E. coli parents.
In a series of eight reciprocal genetic crosses, each hybrid class was remated
with each of the two E. coli Hfr strains. With one exception, recipient ability
was increased in the hybrids only when the E. coli genetic segment previously
integrated matched the proximal region of the remating Hfr chromosome. We
have interpreted this as establishment in S. typhosa of an artificial chromo-
somal homology, which removes the initial barrier to integration of E. coli
lead markers, thereby increasing the integration chances of more distal Hfr
markers.

Discussion

The genetic studies presented in this report confirm and extend the obser-
vations of Luria and Burrous (1957) that E. coli K-12 and Shigella exhibit genetic
homology. Our finding that Shigella hybrids show alterations in virulence as a
consequence of integration of segments of the Escherichia chromosome is most sig-
nificant. Of equal importance is the fact that some hybrids retain virulence
even after incorporation of up to 15 per cent of the Escherichia genome.

The intermediate virulence of those hybrids which had received one of two
widely divergent chromosomal regions is not immediately apparent. However, it
is encouraging that analysis of over one-half of the chromosome has revealed only one region which appears to be essential for the virulence of *S. flexneri* 2a for the guinea pig. The segregation of virulence among the *xyl*<sup>+</sup> and *xyl*<sup>-</sup> *mal*<sup>+</sup> hybrids, and the complete loss of virulence in all hybrids carrying the closely linked *ind*<sup>+</sup> and *rha*<sup>+</sup> *ind*<sup>-</sup> markers indicates that we may be dealing with a single gene, or gene cluster, located between *rha*<sup>+</sup> and *xyl*<sup>+</sup>.

The results obtained with partial diploid strains are strongly suggestive that the observed virulence alterations of *Shigella* hybrids are the direct result of the presence of *Escherichia* genotypic material, rather than some nonspecific phenomenon. In the partial diploid, the hybrid retains its full complement of *Shigella* genes, but is still significantly less virulent than the parental dysentery culture. The strain may return to complete virulence by eliminating the *Escherichia* chromosomal fragment or become avirulent by incorporation of the fragment. The reduced virulence of the diploid cells indicates that one or more determinants of the endogenote important for virulence are recessive to, or may be replaced by, determinants on the *Escherichia* exogenote. In addition, the data obtained with diploids suggest that the virulence loss associated with the incorporation of the *rha*<sup>-</sup> *xyl*<sup>+</sup> region is very likely not due to deletion or unequal crossing over of *Shigella* material.

As indicated previously, evidence is accumulating to show that the chromosomes of *E. coli*, *Salmonella*, and *Shigella* are grossly homologous. This gross homology could indicate that identical chromosomal regions responsible for virulence (or lack of virulence) may be identified in all three groups of organisms. In fact, our preliminary data with *S. typhimurium* indicates that this is the case. Further studies will, of course, be required to verify these
data, and to establish the precise chromosomal location of the genetic determinant (or determinants) responsible for virulence in these organisms. In addition, it is anticipated that the information derived from our studies on *Salmonella* recipient ability will enable us to obtain virulent *Salmonella* and *Shigella* donor strains. The employment of these strains in future studies would provide significant information with regard to virulence, genetic homology, and evolution of species within the *Enterobacteriaceae.*
Literature Cited


Bibliography


### Table 1

Transfer of Genetic Characters from *E. coli* K-12 to *S. flexneri* 2a strain 2457T

<table>
<thead>
<tr>
<th>Cross</th>
<th>Selection</th>
<th>Frequency of Recombination</th>
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<tr>
<td><em>E. coli</em> Hfr W 1895</td>
<td>lac⁺</td>
<td>3 x 10⁻³</td>
</tr>
<tr>
<td>x</td>
<td>ara⁺</td>
<td>5 x 10⁻⁴</td>
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<tr>
<td><em>S. flexneri</em> 2a F- 2457T</td>
<td>rha⁺</td>
<td>3.5 x 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>xyl⁺</td>
<td>2 x 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>mal⁺</td>
<td>2 x 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>fuc⁺</td>
<td>5 x 10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>nic⁺</td>
<td>≤ 10⁻⁷</td>
</tr>
<tr>
<td><em>E. coli</em> Hfr AB 313</td>
<td>xyl⁺</td>
<td>3 x 10⁻³</td>
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<tr>
<td>x</td>
<td>mal⁺</td>
<td>1 x 10⁻³</td>
</tr>
<tr>
<td><em>S. flexneri</em> 2a F- 2457T</td>
<td>nic⁺</td>
<td>5 x 10⁻⁶</td>
</tr>
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<td><em>E. coli</em> Hfr 1362</td>
<td>mal⁺</td>
<td>2 x 10⁻³</td>
</tr>
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<td>x</td>
<td>xyl⁺</td>
<td>1 x 10⁻³</td>
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<td>lac⁺</td>
<td>7 x 10⁻⁵</td>
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The frequency of recombination is expressed as the number of hybrids isolated per donor cell.
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<th>Cross</th>
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<th>No. Examined</th>
<th>lac⁺</th>
<th>ara⁺</th>
<th>pil⁺</th>
<th>rha⁺</th>
<th>ind⁺</th>
<th>xyl⁺</th>
<th>mal⁺</th>
<th>fuc⁺</th>
<th>nic⁺</th>
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<tr>
<td>E. coli W1895 x E. flexneri 2457T</td>
<td>mal⁺</td>
<td>180</td>
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<td>0</td>
<td>9</td>
<td>10</td>
<td>14</td>
<td>--</td>
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<td>sac⁺ - fuc⁺ - lac⁺</td>
<td>1</td>
<td>8/21</td>
<td>38.09</td>
<td>18.30-57.88</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Controls**

| S. flexneri 2457T     | 1                         | 120/157      | 76.43       | 69.79-83.07           |
| S. coli W1895         | 1                         | 1/48         | 2.08        | -1.96-6.12            |
Table 4
Virulence of Partial Diploid Hybrids
Of S. flexneri 2a and Their Haploid Segregants
For the Guinea Pig

<table>
<thead>
<tr>
<th>Genotype of Hybrid</th>
<th>Deaths Total</th>
<th>% Mortality</th>
<th>95% Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{rhe}^{+}\text{-ind}^{+}\text{-xyl}^{+}\text{-mal}^{+} )</td>
<td>11/29</td>
<td>34.48</td>
<td>19.55-49.61</td>
</tr>
<tr>
<td>( \text{rhe}^{-}\text{-ind}^{+}\text{-xyl}^{+}\text{-mal}^{+} )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haploid Segregant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{rhe}^{+}\text{-ind}^{-}\text{-xyl}^{-}\text{-mal}^{-} )</td>
<td>24/29</td>
<td>87.76</td>
<td>63.54-97.82</td>
</tr>
<tr>
<td>Haploid Segregant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{rhe}^{+}\text{-ind}^{+}\text{-xyl}^{+}\text{-mal}^{-} )</td>
<td>2/26</td>
<td>7.69</td>
<td>-2.60-17.98</td>
</tr>
<tr>
<td>( \text{rhe}^{+}\text{-ind}^{+}\text{-xyl}^{-}\text{-mal}^{+} )</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Table 5

Summary of Matings of *S. typhosa* Hybrids with Hfr W1895 and Hfr Hayes

<table>
<thead>
<tr>
<th>Cross</th>
<th>Selection</th>
<th>Mean Freq./Donor Cell</th>
<th>Std. Dev.</th>
<th>Control Cross Freq.</th>
<th>Increase Over Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) W1895 x WSF <em>lac</em>+(95)</td>
<td>arabinose</td>
<td>2.0 x 10^-4</td>
<td>±1.2</td>
<td>1.0 x 10^-6</td>
<td>200X</td>
</tr>
<tr>
<td>(2) W1895 x WSF <em>ara</em>+(95)</td>
<td>lactose</td>
<td>2.1 x 10^-5</td>
<td>±1.8</td>
<td>1.5 x 10^-5</td>
<td>---</td>
</tr>
<tr>
<td>(3) Hayes x WSF <em>ara</em>+(95)</td>
<td>lactose</td>
<td>1.6 x 10^-4</td>
<td>±2.5</td>
<td>6.0 x 10^-7</td>
<td>265X</td>
</tr>
<tr>
<td>(4) Hayes x WSF <em>lac</em>+(95)</td>
<td>arabinose</td>
<td>5.5 x 10^-6</td>
<td>±1.5</td>
<td>4.8 x 10^-6</td>
<td>---</td>
</tr>
<tr>
<td>(5) Hayes x WSF <em>ara</em>+(H)</td>
<td>lactose</td>
<td>4.7 x 10^-5</td>
<td>±3.2</td>
<td>5.0 x 10^-7</td>
<td>94X</td>
</tr>
<tr>
<td>(6) Hayes x WSF <em>lac</em>+(H)</td>
<td>arabinose</td>
<td>8.0 x 10^-5</td>
<td>±3.5</td>
<td>6.0 x 10^-6</td>
<td>13X</td>
</tr>
<tr>
<td>(7) W1895 x WSF <em>lac</em>+(H)</td>
<td>arabinose</td>
<td>4.5 x 10^-5</td>
<td>±2.0</td>
<td>8.2 x 10^-7</td>
<td>55X</td>
</tr>
<tr>
<td>(8) W1895 x WSF <em>ara</em>+(H)</td>
<td>lactose</td>
<td>1.8 x 10^-5</td>
<td>±1.5</td>
<td>1.5 x 10^-5</td>
<td>---</td>
</tr>
</tbody>
</table>

The frequency of the control cross (W1895 or Hayes x 643WSF for the selected marker) represents the highest observed recombination frequency of 3 plates set up concurrently with each of the test crosses.
KEY WORDS DEFINING SUBJECT MATTER FOR INDEXING PURPOSES

1. Enteric bacteria
   - genetics of virulence in

2. Genetics
   - of virulence in enteric bacteria

3. Virulence
   - genetic studies in enteric bacteria