

1 **Genome-wide analysis of DNA uptake by naturally competent *Haemophilus***
2 ***influenzae***

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4 Marcelo Mora^{1¶}, Joshua Chang Mell², Garth D. Ehrlich^{2,3}, Rachel L. Ehrlich² and Rosemary J.
5 Redfield¹

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7 **AUTHOR AFFILIATIONS:**

8 1. Department of Zoology, University of British Columbia, Vancouver, BC, Canada.

9 2. Department of Microbiology & Immunology; Center for Genomic Sciences, Institute of
10 Molecular Medicine and Infectious Disease; Drexel University College of Medicine, 12
11 Philadelphia PA, USA.

12 3. Department of Otolaryngology – Head and Neck Surgery, Drexel University College of
13 Medicine, 12 Philadelphia PA, USA.

14
15 ¶ corresponding author

16
17 **AUTHOR EMAIL ADDRESSES:**

18 MM: mora@zoology.ubc.ca

19 JCM: Joshua.Mell@DrexelMed.edu

20 GDE: ge33@drexel.edu

21 RLE: rle36@drexel.edu

22 RJR: redfield@zoology.ubc.ca.

23

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26 **ABSTRACT**

27 **BACKGROUND**

28 DNA uptake is the first step in natural transformation of bacteria, leading to DNA internalization
29 and recombination. It is, therefore, a key determinant in genome evolution. Most bacteria take up
30 DNA indiscriminately, but in two families of Gram-negative bacteria the uptake machinery binds
31 preferentially to short sequences called uptake signal sequences (USS). These sequences are
32 highly enriched in their genomes, which causes preferential uptake of self-DNA over foreign DNA.

33 **RESULTS**

34 To fully characterize the effects of this preference, and to identify other sequence factors
35 affecting uptake, we carried out a genome-wide analysis of DNA uptake using both measured
36 uptake and the predictions from a sequence-based uptake model. Maps of DNA uptake were
37 developed by recovering and deep sequencing genomic DNA that had been taken up by
38 competent *Haemophilus influenzae* cells, and comparing sequencing coverage from recovered
39 samples to coverage of the input DNA. Chromosomal DNA that had been sheared into short
40 fragments (50-800bp) produced sharp peaks of uptake centered at USS, separated by valleys
41 with 1000-fold lower uptake. Peaks heights were proportional to the USS scores predicted by the

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42 previously measured contribution to uptake of individual bases in each USS, as well as by
43 predicted differences in DNA shape. Uptake of a long-fragment DNA preparation (1.5-17kb) had
44 much less variation, with 90% of positions having uptake within 2-fold of the mean. Although the
45 presence of a second USS within 100bp had no detectable effect on uptake of short fragments,
46 uptake of long fragments increased with the local density of USS. Simulation of the uptake
47 competition between *H. influenzae* DNA and the abundant human DNA in the respiratory tract
48 DNA showed that the USS-based system allows *H. influenzae* DNA to prevail even when human
49 DNA is present in 100-fold excess.

50 CONCLUSION

51 All detectable DNA uptake biases arose from sequences that fit the USS uptake motif, and
52 presence of such sequences increased uptake of short DNA fragments by about 1000-fold.
53 Preferred sequences also had rigidly bent AT-tracts and outer cores. Uptake of longer DNA
54 fragments was much less variable, although detection of uptake biases was limited by strong
55 biases intrinsic to the DNA sequencing process.

56 Keywords

57 DNA uptake, uptake bias, natural transformation, competence, uptake signal sequences, deep
58 sequencing.

59

60

61 Introduction

62 Many bacteria are naturally competent, able to actively bind DNA fragments at the cell surface
63 and pull them into the cytoplasm, where the incoming fragments may contribute nucleotides to

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64 cellular pools or recombine with homologous genomic sequences (1). The genetic exchange
65 associated with this latter process contributes to adaptation and is known to have promoted
66 resistance to antibiotics (2) and increased strains' intracellular invasiveness (3) and vaccine
67 resistance (4,5). Thus, understanding how different genomic regions evolve via natural
68 transformation processes could be used to predict the spread of pathogenic traits.

69 Most competent bacteria that have been tested take up DNA regardless of sequence, but species
70 in two families, the Pasteurellaceae and the Neisseriaceae, exhibit strong sequence biases for
71 short motifs (6). Because these motifs have become highly enriched in the corresponding
72 genomes, these biases effectively limit uptake to DNA from close relatives with the same uptake
73 specificity (7,8). The distribution of the preferred sequences around the chromosome is uneven
74 (9), which may cause different genes to experience quite different rates of genetic exchange.

75 Most steps in the DNA uptake process are highly conserved among naturally transformable
76 species (6). In the Pasteurellaceae, the Neisseriaceae and most other Gram-negative bacteria,
77 DNA uptake is initiated by binding of a type IV pilus uptake machine to dsDNA at the cell surface.
78 This is followed by the retraction of the pilus, which pulls the DNA across the outer membrane
79 into the periplasm. Uptake is thought to begin internally on DNA fragments, not at an end,
80 because circular DNAs are taken up as efficiently as linear DNAs (10). Thus, it is likely that the
81 stiff dsDNA molecule is transiently kinked (folded sharply back on itself) at the site of initiation to
82 allow it to pass through the narrow secretin pore of the uptake machinery. Forces generated by
83 the retraction of the type IV pilus are thought to be responsible for this kinking. Once a loop of
84 the DNA is inside the periplasm, a ratchet process controlled by the periplasmic protein ComEA is
85 thought to pull the rest of the DNA through the outer membrane (11,12). Subsequent
86 translocation of the DNA into the cytoplasm requires a free DNA end; only the 3'-leading strand
87 passes through an inner membrane pore encoded by the *rec2/comEC* gene, while the other

88 strand is degraded and its nucleotides dephosphorylated and imported as nucleosides (13).

89 Circular DNA molecules are not transported from the periplasm into the cytoplasm because they

90 lack free ends (13).

91 **Direct measures of DNA uptake bias:** Uptake-competition experiments in the Pasteurellacean

92 *Haemophilus influenzae* and in *Neisseria gonorrhoeae* showed that uptake of genetically marked

93 ‘self’ DNA was inhibited by unmarked self DNA but not by DNA from unrelated sources (7,8).

94 Subsequent DNA uptake experiments using cloned radiolabeled DNA fragments found that the *H.*

95 *influenzae* self-preference is caused by the uptake machinery’s strong bias for a short sequence

96 motif, the uptake signal sequence (USS) (14). Sequence comparisons and site-directed

97 mutagenesis initially identified an 11bp sequence, with a strong contribution by flanking AT-rich

98 sequences (15,16), and genome sequencing identified 1465 occurrences of a 9bp USS core in *H.*

99 *influenzae*, and 1892 occurrences of an unrelated 10bp ‘DUS’ in *N. meningitidis* (9,17). Later

100 analyses using mutagenesis and sequencing of pools of degenerate USS identified the

101 contribution of each position, which are summarized by the sequence logo in Figure 1 (18,19).

102 This study found the central GCGG bases to be crucial for uptake, with smaller and synergistic

103 contributions made by flanking bases and two adjacent AT-rich segments. The Pasteurellacean

104 USS is unrelated to the Neisseriacean DNA uptake sequence (20), and different lineages within

105 each of the families can have variant preferred motifs (21,22).

106 **Evolution of uptake sequences in the genome:** Alignment of distinct homologous genomic

107 regions between distantly related Pasteurellaceae species showed that the USS evolve by point

108 mutations (22); *i.e.* they are not insertion elements. Danner et al. (15) proposed that the

109 combination of uptake bias and genomic recombination creates an evolutionary pressure that

110 will cause the preferred uptake sequences to accumulate throughout the genome, with locations

111 limited mainly by interference with gene functions. Consistent with this, both USS and DUS are

112 underrepresented in newly acquired segments, in rRNA genes, and in coding sequences,
113 especially those with strong functional constraints (17,23). Modeling by Maughan et al. (24)
114 confirmed that this molecular drive process could produce uptake sequence distributions like
115 those of real genomes, with no need for direct selection for these sequences or for the
116 chromosomal recombination they promote. Thus, the presence of biased DNA uptake machinery
117 may be sufficient in itself to explain the abundance of uptake sequences. Such sequence biases
118 may have arisen solely by direct selection on the DNA uptake machinery for more efficient DNA
119 binding, or by this in combination with indirect selection for preferential uptake of conspecific
120 DNA.

121 Pasteurellaceae and Neisseriaceae species occur primarily in respiratory tracts and other
122 mucosal environments (25), where transformation can only occur if the released bacterial DNA is
123 able to compete with abundant host DNA for binding to the uptake machinery (26,27). These host
124 DNAs are not expected to be enriched for uptake sequences and, since recombination requires
125 strong sequence similarity between incoming DNA with a genomic segment, any nonhomologous
126 DNA sequences that are taken up will usually be degraded rather than recombining with the
127 bacterial genome (28).

128 The goal of the present study was to measure DNA uptake at every position in the *H. influenzae*
129 genome, and to use this data to characterize the DNA uptake biases caused by the USS and any
130 other sequence factors. We first developed a computational model that predicted the effect of
131 uptake sequences on DNA uptake across the *H. influenzae* genome. This model's predictions were
132 then compared with actual measurements of DNA uptake produced by sequencing genomic DNA
133 fragments that had been recovered after being taken up by competent *H. influenzae* cells.
134 Discrepancies between predicted and observed uptake revealed the strength of the bias, effects
135 of USS sequence differences, and the influence of the distribution of USS locations. These factors

136 in turn increase the understanding of the genomic distribution of recombination and the effects
137 of competition with DNA from the host or other microbiota.

138

139 **Results**

140 **A computational model of DNA uptake:**

141 As a framework for interpreting DNA uptake data we developed a simulation model of USS-
142 dependent DNA uptake. It takes as input the locations and strengths of USSs in the DNA whose
143 uptake is to be simulated, the fragment-size distribution of this DNA, and an uptake function that
144 describes how uptake probability depends on USS presence and strength. The output is the
145 expected relative uptake of every position in the genome.

146 In developing the model we were guided by basic principles of how sequence-specific DNA-
147 binding proteins interact with DNA (29,30). The first step in these interactions is thought to be a
148 random encounter between a DNA fragment and the binding site of the protein, usually at a DNA
149 position that does not contain the protein's preferred sequence. This non-specific binding
150 dramatically increases the probability that the protein will subsequently encounter any preferred
151 sequence, either by sliding along the DNA or by transient dissociation and reassociation, leading
152 to specific binding between DNA and protein. In the case of the USS this specific binding enables
153 uptake of the DNA fragment across the cell's outer membrane.

154 The model did not explicitly simulate the first step, non-specific binding, since this is expected to
155 be equally probable for all DNA positions. The specific binding and DNA uptake steps were
156 separately modeled since they are expected to depend on the properties of the DNA uptake
157 machinery and on the length and sequence of the DNA fragment. Although in real cells both steps

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158 may depend on the quality of the USS, for simplicity the initial version of the model assumed that
159 specific binding required only a threshold similarity to the USS consensus, and that the
160 subsequent probability of uptake depended on the strength of this similarity.

161 Simulating these steps required first specifying the genomic sequences that should be treated as
162 USS. This was not straightforward because genomes contain many USS variants that differ in how
163 well they promote DNA uptake (18,23). Our strategy was to score genome positions with the
164 uptake-prediction matrix from Mell et al.'s degenerate-sequence uptake experiment (19) (Table
165 S1), and to use overrepresentation of high-scoring sequences as the USS criterion. We scored
166 every position in the genomes of the standard *H. influenzae* reference strain Rd, of the two strains
167 whose uptake we investigated, 86-028NP ('NP') and PittGG ('GG'), and of four randomly
168 generated genome-length sequences with the same base composition (Supp Figure 1). In the *H.*
169 *influenzae* genomes, overrepresentation of high-scoring sequences was detectable above a score
170 of 7.0 bits and became dramatic above 10.0 bits, where the numbers of high-scoring positions
171 increased in *H. influenzae* genomes but became vanishingly small in the random-sequence
172 controls, (see inset in Supp. Figure 1). DNA uptake analyses used a USS cutoff score of 10 bits
173 ('USS₁₀', n=1941) or a less stringent 9.5 bits ('USS_{9.5}', n=2248).

174 The binding step of the computational model evaluated whether the fragment under
175 consideration contains any USS₁₀, and their probability of being encountered by the uptake
176 machinery receptor. In Model version I, fragments with no USS had a baseline binding probability
177 of 0.2; this was reduced in Model versions II and III. In Model versions I and II the encounter
178 probability decreased linearly with fragment length, but increased if more than one USS₁₀ was
179 present in proportion to their separation. In Model III binding was instead a function of the
180 number of USS₁₀ in the fragment. The probability that this binding led to DNA uptake was a
181 function of the USS score, from a baseline of 0.2 at a score of 9 bits to a maximum of 1 at 12.6 bits.

182 In Model version 1 this function was linear, but it was replaced with a sigmoidal function in
183 Models II and III.

184 Once the contributions of every size class of fragment had been calculated for each position
185 (Figure 2A), the model combined all the contributions, taking into account the frequency of each
186 size class in the input DNA. The position-specific uptake predictions were then normalized to a
187 genome-wide mean uptake probability of 1.0.

188 **Model results:** Figure 2B and C show examples of model predictions for simple situations.
189 Figure 2B shows the uptake predictions for an 800-bp simulated genome containing a single USS
190 with score 12.0 bits, considering three different input DNA fragment sizes (100, 200 and 300bp).
191 The peaks at the USS have straight sides, a basal width twice the length of the fragments being
192 taken up, and 31-bp flat tops arising from the model's requirement for a full-length USS. When
193 the DNA fragment sizes were evenly distributed between 25-300bp in length (Figure 2C and D),
194 the peak had steep sides at its tops and gradually flattened at the base; maximum width at the
195 base equaled twice the maximum fragment length. The grey peak in Figure 2C shows that model
196 versions with a baseline of USS-independent uptake caused valleys to be higher and peaks
197 correspondingly lower. With this original version of the model ('Model I' Table S4), heights of
198 predicted peaks were linearly proportional to USS scores (dashed red line in Figure 2D). In
199 simulated genomes with more than one USS (Figures 2D, 2E, 2F), isolated peaks were only seen
200 when the DNA fragments being taken up were substantially shorter than the spacing of the USSs,
201 and disappeared entirely when the fragments were long enough that almost all contained at least
202 one USS (Figure 2F).

203 **Figure 2G and 2H** show the predicted uptake maps when this model analyzed a 50kb segment of
204 the *H. influenzae* NP genome, using the short-fragment and long-fragment size distributions from

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205 the actual uptake experiments described below (Supp. Figure 2A and B), and Figure 2I shows the
206 distribution of USSs over this segment. Because the short DNA fragments are shorter than the
207 typical separation between USSs, uptake is predicted to be restricted to sharp peaks at each USS.
208 In contrast, uptake of long DNA fragments is predicted to be much more uniform, since most of
209 these will contain at least one USS.

210 **Generation of experimental DNA uptake data:**

211 To obtain high-resolution measurements of actual DNA uptake we sequenced *H. influenzae*
212 genomic DNA that had been taken up by and recovered from competent *H. influenzae* cells.
213 Competent cells of the standard laboratory strain Rd were first incubated with genomic DNA
214 preparations from strains NP and GG, whose core genomes differ from Rd and each other at ~3%
215 of orthologous positions (31). To allow efficient recovery of the taken-up DNA, the Rd strain in
216 which competence was induced carried a *rec2* mutation that causes DNA to be trapped intact in
217 the periplasm (16). The NP and GG genomic DNAs were pre-sheared to give short (50-800bp)
218 and long (1.5-17kb) DNA preparations (size distributions are shown in Supp. Figure 2), and three
219 replicate uptake experiments were done with each DNA preparation. After 20 min incubation
220 with competent cells, the taken-up DNA was recovered from the cell periplasm using the cell-
221 fractionation procedure of Kahn et al. (19,32,33). Recovered DNA samples were sequenced along
222 with samples of the input NP and GG DNAs and of the recipient Rd DNA. The input and uptake
223 reads were then aligned to the corresponding NP and GG reference sequences and coverage at
224 every position was calculated. Table S2 provides detailed information about the four input
225 samples, the twelve uptake samples, and the Rd sample.

226 **Effects of contaminating Rd DNA:** Preparations of DNA recovered after uptake always included
227 some contaminating DNA from the recipient Rd chromosome. The divergence between the Rd

228 and donor genomes allowed the extent of this contamination to be estimated by competitively
229 aligning the recovered reads from each sample to a reference that included both recipient and
230 donor genomes as separate chromosomes. Thus, reads that uniquely aligned to only one
231 chromosome could be unambiguously assigned to either donor or recipient. The resulting Rd
232 chromosomal contamination estimates were between 3.2% and 19.3% of reads; specific values
233 for each sample are listed in Table S2.

234 The effects of this contamination were not expected to be uniform across the donor genome,
235 since segments of the NP and GG genomes with high divergence from or with no close homologs
236 in Rd would be free of contamination-derived reads. We used the competitive-alignment
237 described above to create contamination-corrected uptake coverages, by discarding all reads that
238 preferentially aligned to Rd rather than NP or GG. We also discarded reads that could not be
239 uniquely mapped to the donor genome; this included reads from segments that are identical
240 between the two strains ('double-mapping reads') and reads that mapped to repeats, such as the
241 six copies of the rRNA genes. This removed an average of 18.6% of reads (range 8.9%-28.3%),
242 left some segments of the NP and GG genomes with no coverage in all samples (2.3% and 2.1%
243 respectively) and reduced coverage adjacent to these segments. Contamination details for each
244 sample are provided in Supplementary Table 2, and the impacts are considered below.

245 **Uptake ratios:** To control for position-specific differences in sequencing efficiency, read
246 coverage at each position in each uptake sample was divided by read coverage in the
247 corresponding input sample (e.g. each NP-short uptake sample by NP-short input). Normalizing
248 the mean of the three replicates to a genome-wide mean uptake of 1.0 then gave a mean 'uptake
249 ratio' measurement for each genome position for each DNA type. Figure 3 and Supp. Figure 3
250 show the resulting uptake ratio maps, smoothed using a 31bp sliding window.

251 Figure 3A shows the short-fragment uptake ratio map for the first 50kb of the NP genome; the
252 ticks in Figure 3C indicate locations and scores of USS_{10S}. The pattern is strikingly similar to that
253 predicted by the model (Figure 2G). Sharp uptake peaks are seen at USS₁₀ positions; some peaks
254 are separated by flat-bottomed valleys and others overlap. Supp. Figure 3A shows a similar map
255 for the first 50kb of strain GG's genome. The full-genome maps of these NP and GG uptake ratios
256 in Supp. Figure 3D and 3G display the consistency of the peak heights.

257 Also as predicted by the model, the long-fragment DNA samples (Figure 3B and Supp. Figs 3B, E
258 and H) had much less variation in uptake than the short-fragment samples; 90% of positions had
259 uptake ratios within two-fold of the mean, and there were few high peaks or low valleys.
260 Extended genome segments with low or no uptake coincided with large gaps between USS_{10S}.
261 The largest gap is in the NP segment between 95 and 145kb —the site of a genomic island with
262 high similarity to an *H. influenzae* plasmid but few USS (34).

263 **Sources of variation:** Characterization of USS dependent uptake biases and possible USS-
264 independent biases in uptake coverage was limited by strong variation in sequencing coverage,
265 presumably due to biases in the library preparation and sequencing steps. Supp. Figure 4A
266 compares coverage for the NP short and long input samples, showing that this variation was both
267 reproducible and sequence dependent. These biases are expected to have very similar effects on
268 coverage in all samples, precluding calculation of uptake ratios where input coverage is zero and
269 generating high levels of stochastic variation where coverage is low.

270 In Supp. Figure 4B and C, the colouring of NP long-fragment uptake ratio points according to
271 input coverage reveals that all of the extreme uptake ratio values occurred in regions of low input
272 coverage. Table S3 extends this analysis to the whole genome, showing that anomalously high
273 uptake was seen mainly at positions with low input coverage, indicating that these values are

274 likely due to stochastic variation rather than to genuinely high uptake. In contrast, positions with
275 low uptake showed no such bias, indicating that these are mainly due to genuinely low uptake.

276 **Periodicity:** Bacterial genomes show periodicity for several features related to DNA curvature
277 and codon usage biases (35), so we examined the distribution of uptake ratios across each
278 genome by Fourier analysis, using the R package TSA. The log-log views in Supp. Figure 5 show
279 that this found no strong influence of any specific repeat period on either the variation in input-
280 sample coverage (panels A-D) or the variation in uptake ratios (panels E-H). Instead, to explain
281 the observed variation the analysis needed to invoke small contributions from almost every
282 possible repeat period.

283 **Uptake bias analysis:** Our strategy to investigate the DNA uptake process was to analyze
284 discrepancies between model predictions and observed uptake ratio peaks in the NP short-
285 fragment dataset, since these revealed ways in which the simple assumptions underlying the
286 model mis-characterized the actual steps of DNA uptake. Model changes that improved the
287 predictions were considered to better reflect the true constraints on uptake of short DNA
288 fragments. We then compared the refined model's predictions to the real uptake ratios for the NP
289 long-fragment DNA, and finally to the long- and short-fragment uptake ratios for the GG DNA.
290 Figure 4A compares predicted (orange line) and measured (blue line) uptake of short-fragment
291 DNA for the first 50kb of the NP genome. The model's predictions of peak locations and peak
292 shapes were both extremely accurate, but the predicted baseline uptake in the valleys between
293 peaks was too high, and some predicted peaks were too high or too low.

294 We next inspected the depths of the valleys between uptake ratio peaks. Although these were
295 quite variable, (see log-scale inset in Figure 3A), the histogram of uptake ratios below 0.1 in Supp.
296 Figure 6 shows that most deep valleys fell to uptake ratios between 0.0005 and 0.005. In Model

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297 version I, fragments that lacked USS were arbitrarily assigned binding and uptake probabilities of
298 0.2, resulting in predicted baseline uptake of ~ 0.08 . To improve the model, we lowered the both
299 baseline parameter settings from 0.2 to 0.02, which gave predicted baseline uptake of ~ 0.002 in
300 regions far from a USS.

301 The peak heights predicted by the initial model were linearly proportional to USS score (Figure
302 2D) reflecting the model's assumed linear dependence of uptake probability on USS score (blue
303 line in Figure 4B). However, analysis of 209 USS_{9.5} that were separated by at least 1000 bp (to
304 minimize effects of overlapping peaks) (black dots in Fig. 4B) showed that experimental uptake
305 ratios instead followed a sigmoidal relationship with score. Very little uptake was seen at
306 isolated USSs with scores between 9.5 and 10 bits, and consistently high peaks were observed at
307 isolated USSs with scores above 11.5 bits. Accordingly, Model version I was further revised to use
308 a sigmoidal function fit to this data (orange line in Figure 4B); the new predictions (Model
309 version II) better matched the observed valley depths and peak heights (Figure 4C; Pearson
310 correlation rose from 0.691 to 0.755).

311 **Symmetry and shape of uptake peaks:** The DNA uptake motif is not palindromic, so
312 asymmetric interactions of DNA with the uptake machinery could polarize DNA uptake by
313 causing one side of the motif to be pulled into the cell more efficiently than the other. The motif's
314 strongly weighted positions are also all on one side, not at its center, which might cause peak
315 centers to be shifted relative to USS centers. Supp. Figure 7 shows that, when all isolated USS_{10.5}
316 with high uptake (≥ 3) were analyzed in the same orientation, the mean peak was both centered
317 on the USS and symmetric about it (no significant difference between mean ratios left and right of
318 the USS center at position 16 ($p=0.9$)).

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319 **Pairwise base interaction:** Mell et al. (19) found evidence for substantial contributions to
320 uptake by long-distance pairwise interactions between AT-tract bases and core bases. To
321 incorporate the effects of these interactions in the model, USS scores were adjusted using the
322 interaction information in Figure 6 of Mell et al. (19). This change had little effect on high-scoring
323 USS, but further reduced the scores of low-scoring USS (Supp. Figure 8). The uptake ratios
324 predicted by the modified scores were no more accurate than those for the original scores
325 (correlation between observed uptake and predicted uptake without interactions: 0.937; with
326 interactions: 0.936), likely because most of the affected scores were already very low (70% were
327 < 10.5).

328 **DNA shape effects:** Although the analysis of Mell et al. (19) found no evidence of pairwise
329 interactions between close positions, we used analysis of DNA shape to detect both pairwise and
330 more complex interactions over a 5bp distance. Shape features that can be predicted from DNA
331 sequence includes the minor groove width, the propeller twist between bases in a base pair, the
332 helix twist between one base pair and the next, and roll, the rotation of one base pair relative to
333 the next. The thick grey line in each panel of Figure 5 shows these features for the consensus
334 USS. The USS inner core (orange shading) has a relatively wide minor groove and high propeller
335 twist, which would facilitate sequence recognition by proteins (36). To the left of this and in both
336 AT-tracts (yellow shading) the minor groove is narrow with low propeller twist and negative roll,
337 predicting that these segments are both rigid and slightly bent.

338 The coloured lines in Figure 5 compare the shape features of subsets of isolated USS with similar
339 scores but different uptake ratios. Panels A-D compare the shape features of low-scoring USS
340 ($USS_{10-10.5}$) whose uptake ratios were low (<0.6, blue lines) or high (>2.0, orange lines). Similarly,
341 panels E-H show the same comparison for USSs with better scores ($USS_{10.5-11}$). USSs with scores
342 higher than 11 were not analyzed since they did not exhibit enough uptake variation to reveal

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343 correlations between uptake and DNA shape. Although very similar inner-core shape features
344 were seen for low-uptake and high-uptake subsets, the AT-tract shapes had marked differences,
345 with low-uptake USSs having no distinctive shape features and high-uptake USSs resembling the
346 USS consensus shape. This suggests that the predicted rigidity and slight bend of the AT tracts
347 facilitate DNA uptake.

348 **Detecting weak uptake biases:** Any weak uptake biases that exist will only be detectable in
349 genome segments that lack a strong USS, so we searched for biases arising from either low-
350 scoring USS or other factors using a far-from-USS₁₀ dataset containing only DNA segments whose
351 ends were at least 0.6kb from the closest USS₁₀. This dataset contained 575 segments where
352 weak uptake effects could in principle be detected (29% of the genome); their mean uptake ratio
353 was 0.0097. Of these segments, 62 were set aside because they had low input coverage (<20
354 reads). Only 16 of the remaining 513 segments contained positions with uptake ratios >0.2,
355 indicating that sequences conferring weak biases are quite rare. Ten of these segments contained
356 distinct peaks (heights between 0.2 and 1.0) that coincided with weak USSs scoring between 9.47
357 and 10 bits; the other six lacked distinct peaks but contained shoulders at the extended bases of
358 strong USS₁₀ peaks. However, this far-from-USS₁₀ dataset also contained 68 other similarly weak
359 USS that were not associated with uptake peaks (scores 9.50-9.99 bits, mean uptake of 0.033).
360 Panels I-L of Figure 5 show that shapes of the 10 USS with uptake > 0.2 (orange lines) were more
361 similar to that of the consensus USS than the shapes of the 68 USS with no peaks (blue lines). The
362 boxplot in Supp. Figure 9 summarizes uptake ratios at these 78 weak USSs, showing that median
363 uptake ratios were very low for all sub-classes of weak USSs. Since this analysis did not find any
364 non-USS positions giving uptake higher than 0.2, it also shows that other sequence factors do not
365 detectably promote uptake in the absence of a USS.

366 **Uptake of fragments with multiple USSs:** Fragments containing two or more uptake sequences
367 might be expected to have higher uptake, since they have more targets to which the uptake
368 machinery receptor could bind, but only one of the two previous studies in *Neisseria* found this
369 effect (37,38). Many genomic USSs are sufficiently close that they will co-occur even on short
370 DNA fragments; 23% of NP USS_{10s} are within 100bp of another USS₁₀, and 17% are within 30bp
371 (Supp. Figure 10A). The initial runs of the uptake model assumed that multiple USS on the same
372 fragment decreased the search distance for the specific-binding step but did not affect the uptake
373 step, which used the mean USS score, not the best. This predicted single peaks at pairs of USS₁₀
374 within 100bp of each other, and two distinguishable peaks or a peak with a distinct shoulder at
375 USS with wider separations. Except for very close USSs, the single peaks were about 15% higher
376 than for isolated USSs with the same scores.

377 Visual examination of uptake ratios at the 230 pairs of NP USS_{10s} within 100bp found single
378 peaks; Supp. Figure 10B) shows that these USS pairs (coloured points) do not have noticeably
379 higher uptake ratios than isolated USSs (grey points, from Figure 4). However, a mean difference
380 in peak heights of less than 12% could not be confidently detected because of the low numbers of
381 USS pairs, especially those with scores lower than 11.0 bits. A special class of USS pairs consists
382 of overlapping oppositely oriented pairs that are located at the ends of genes and act as
383 transcriptional terminators (9,17,39). Supp. Figure 10A shows that the NP genome has 109 USS₁₀
384 pairs whose centers are within 14bp: 69 0-3 bp apart (-/+ orientation) and 40 10-14bp apart (+/-
385 orientation). Supp. Figure 10C shows that uptake ratios at these did not differ from those at
386 isolated USS_{10s} (P = 0.12 for the 103 pairs whose mean scores were ≥ 11.0 bits). These results
387 suggest that the presence of two USS_{10s} in a 100bp segment does not detectably increase the
388 probability of the receptor finding a USS, a result consistent with that of Ambur et al. (37).

389 **Uptake of long-fragment NP DNA:**

390 Next step was to investigate the uptake of longer DNA fragments, using the improved model
391 (Model II, Table S4) and the NP long-fragment dataset. Supp. Figure 11A compares this model's
392 predictions with the observed uptake over the same 50kb genome segment as in Figure 3B. In
393 contrast to the model's accurate prediction of short-fragment uptake ratios (correlation of 0.94),
394 it seriously underpredicted the variation in long-fragment uptake ratios (correlation of 0.61),
395 predicting uptake <0.8 or >1.2 for only 13% of NP positions when experimental uptake ratios
396 were outside these limits at 51% of positions. The likeliest explanations are that: 1) the
397 fragment-length distribution the model used overestimated the actual proportions of long
398 fragments available for uptake, or 2) USS density has an effect on uptake of long fragments that
399 was not detectable in the short-fragment dataset.

400 To investigate the first explanation, uptake predictions were generated using a shorter fragment-
401 length distribution, that of NP-long DNA recovered after uptake. Although this DNA's substantial
402 depletion of long fragments (Supp. Figure 12) could be due to non-uptake effects (post-uptake
403 steps in the periplasm or biases during DNA purification), it could also be due to preferential
404 binding or uptake of short fragments. This length distribution thus provided a lower-bound
405 estimate on the real sizes of fragments that were taken up. However, when it replaced the input
406 DNA distribution as a parameter in Model II, the model's correlation with observed uptake ratios
407 was only slightly improved (0.63 vs 0.61) (Supp. Figure 11B), suggesting that fragment length
408 differences were not a major factor.

409 To test the second explanation, we revisited the effect of multiple USS on uptake, this time
410 examining the relationship between DNA uptake and the number of USS_{10s} in a 5kb
411 neighbourhood. Supp. Figure 13A shows that positions with higher local USS densities had higher
412 uptake, and that Model version II only partially accounted for this effect (compare black and red
413 lines). Since mean USS scores did not increase with numbers of USS in the window (Supp. Figure

414 13B), long fragments with more USSs must instead have a higher uptake probability than
415 predicted by the model. The model was consequently revised again; rather than using fragment
416 length and USS separation to calculate binding of each fragment, Model version III used the
417 observed relationship between USS density and uptake ratio (black line in Supp. Figure 13A) to
418 specify fragment-binding probabilities as a function of the number of USS in the fragment.
419 However, this only slightly improved the USS density analysis and the overall correlation
420 between predicted and observed uptake (Supp. Figures 11C and 13C; correlation 0.65 vs. 0.61),
421 and caused a corresponding decrease in the short-fragment correlation (0.90 vs. 0.94).
422 Predictions were slightly better when the recovered fragment-length distribution was used with
423 Model III, (Supp. Figure 13A, green line; overall correlation =0.67), suggesting that both
424 explanations contribute to the uptake variation.

425 Because the correlation between USS-based prediction and observation was only modestly
426 improved by these changes, we also investigated the extent to which the correlation was limited
427 by stochastic noise arising at the regions of low sequencing coverage described earlier. To
428 estimate the magnitude of this effect, we compared the effects of adding different amounts of
429 artificially generated noise to simulated (noise-free) uptake data. Supp. Figure 14 shows that
430 although the correlation between noisy and noise-free data worsened as the arbitrary level of
431 noise increased for both short-fragment (blue) and long-fragment (red) simulations, the effect
432 was much worse for the long-fragment simulations. Simulations with noise levels of 2 and 2.5
433 gave short-fragment correlations very close to the 0.90 between the model and the real data. For
434 the same noise levels the long-fragment correlations were 0.86 and 0.75 respectively, confirming
435 that much of the disparity between measured uptake ratios and USS-based predictions was due
436 to noise in the data. However, these correlations are still 21% and 10% higher than the best

437 correlation obtained between Model III predictions and real data, suggesting that one or more
438 factors remain unidentified.

439 **How well does the model predict uptake of PittGG DNA?**

440 Since the final version of the model (Model III) had been refined using uptake data for DNA of
441 strain 86-028NP, we further evaluated it using the measured uptake data for DNA of strain
442 PittGG, which differs from NP by SNPs and indels affecting about 11% of its genome. Supp. Fig. 15
443 compares the model's uptake predictions with the observed GG uptake ratios. For short-fragment
444 data the correlation between predicted and observed uptake of GG DNA was 0.90, the same as
445 that for NP. However, for long-fragment data, the GG correlation was substantially worse (0.50
446 compared to 0.65 for NP).

447 Some of this discrepancy is due to noise arising from low sequencing coverage. For GG DNA the
448 mean uptake ratio was substantially greater, and the variation more extreme, at low coverage
449 positions (Supp. Figure 16A); this was not seen for NP DNA. However, the cause of this is not
450 clear, since NP and GG had similar frequencies of low-coverage positions for both short-fragment
451 and long-fragment input samples (Table S3).

452 **Predicted competition with human DNA:**

453 *H. influenzae*'s natural environment is the human respiratory tract, where *H. influenzae* DNA must
454 compete for uptake with host-derived DNA whose mucus concentration can exceed 300 µg/ml in
455 healthy individuals (26,27). We used the final model (version III) to investigate this competition.
456 We started by scoring the human genome for USS. This identified 14924 USS_{10s} (density 4.6/Mb),
457 with a mean score of 10.29 bits. For comparison, the NP genome has 1022 USS_{10s}/Mb with a
458 mean score of 11.45 bits, and simulated sequences with the 41% GC content of human DNA had
459 56 USS_{10s}/Mb with a mean score of 10.31 bits. Since the USS motif includes a CpG, the

460 underrepresentation of USS₁₀ in human DNA is probably a consequence of the 4-5 fold depletion
461 of CpGs in the human genome due to deamination of methylated cytosines (40,41).

462 To determine how much human DNA would be needed to outcompete *Haemophilus* DNA for
463 uptake, we first determined the proportion of expected uptake from USS₁₀ in human vs
464 *Haemophilus* genomes. We did this by using Model III to predict uptake of the *H. influenzae* NP
465 genome and of 4 randomly selected 1.9 Mb segments of human DNA. Because human DNA will
466 contain many fragments lacking USSs the predictions were made using baseline binding and
467 uptake probabilities of 0.0 and 0.02. To approximate the lengths of DNA fragments in the
468 respiratory tract (26,27), the model was run using fixed fragment lengths of 1kb and 10kb. The
469 predicted uptake at each position (without normalization) was then summed across all positions
470 to get a total uptake value for each fragment length and baseline assumption. Table S5 shows
471 these uptake values, in arbitrary units.

472 In all cases, if *H. influenzae* cells were exposed to an equal mixture of *H. influenzae* and human
473 DNAs, more than 99% of the DNA taken up was predicted to be from *H. influenzae*. Substantial
474 amounts of *H. influenzae* DNA (14-35%) would be taken up even if the human DNA were in 1000-
475 fold excess. For 10 kb fragments, baseline uptake of fragments lacking USS made only a small
476 contribution, but for 1 kb fragments it increased total uptake of human DNA by 86%, and reduced
477 *H. influenzae* DNA's advantage by 36% when human DNA is in 1000-fold excess. The uptake
478 advantage of *H. influenzae* DNA is due to both the much higher frequency of USS₁₀s in its genome
479 and to its USSs' much stronger matches to the USS consensus.

480 **Discussion**

481 We measured DNA uptake by competent *H. influenzae* cells at every position in the genome, using
482 short-fragment and long-fragment DNA preps from two divergent strains. Differences between

483 predicted and observed uptake revealed the strength of the uptake machinery's bias towards
484 USS, the absence of other sequence biases, and a role for DNA shape. These findings increased
485 our understanding of DNA uptake bias and the role it plays in recombination.

486 **Implications for the molecular mechanism of DNA uptake.**

487 The uptake specificity for USS is very strong. With short fragments, valleys at USS-free segments
488 had ~1000-fold lower uptake ratios than peaks at USS. Although the non-zero uptake ratios in
489 USS-free regions could mean that fragments lacking USS are occasionally taken up, they are also
490 consistent with no uptake at all of fragments lacking a USS, since this low coverage could have
491 arisen artefactually, either from low-level contamination of the recovered DNA with 0.2%-0.8%
492 donor DNA that had not been taken up, or from under-correction of the contamination of
493 recovered-DNA samples by recipient DNA.

494 The correlation of the model predictions with measured uptake ratios was excellent for short
495 fragments but modest for long fragments. However, the model's predictions may be more
496 accurate than indicated by the correlation coefficients, since stochastic variation at low coverage
497 positions introduced substantial noise into the calculation of experimental uptake ratios. Similar
498 errors associated with changes in coverage have been detected in ChIP-seq and RNAseq studies
499 (42-44).

500 Previous analyses of the effects of multiple uptake-sequences on the amount of DNA taken up
501 gave seemingly contradictory results, which ours help resolve. Consistent with Ambur et al.'s
502 (37) study of very close uptake-sequences in *Neisseria*, we did not detect any increased uptake
503 when a second USS₁₀ was within 100 bp of the first. Consistent with Goodman and Scocca's (38)
504 results, also in *Neisseria*, we found that, for larger DNA fragments, a higher local density of USS
505 gave higher uptake. Since DNA-binding proteins can search for their sequence-target by 1-

506 dimensional sliding (30), this discrepancy might arise from the effect of fragment length and USS
507 number on the chance that the uptake receptor will detach from the fragment without having
508 found a USS. Density of USSs might thus have a greater impact in long fragments where the
509 probability of detaching will be greater.

510 The predicted shape differences between USSs with strong or weak uptake suggest strong uptake
511 bias for USS that are rigidly bent at AT-tracts and outer core (36,45). Similar preferences have
512 been described for several DNA binding proteins and have been associated with specific binding
513 by arginine or lysine residues to narrow minor grooves (36,46). These features have been
514 integrated successfully in some transcription factor binding models (47), but using them to
515 improve uptake prediction will require more comprehensive investigation into the effects of DNA
516 shape on uptake.

517 **Implications for recombination.**

518 Davidsen et al. (48) found higher densities of USS in genes for DNA replication, repair and
519 recombination, and suggested that this distribution resulted from selection for preferential
520 recombination of genes involved in maintenance of the genome. However, any effects of DNA
521 uptake biases on the distribution of recombination across the *H. influenzae* genome are likely to
522 be weak. Although uptake of short DNA fragments (<800 bp) depends dramatically on USSs, this
523 will have little genetic consequence since such short fragments typically are degraded before
524 they can recombine (13). On the other hand, 96% of fragments long enough to participate
525 efficiently in recombination (~3.5kb) contain at least one USS₁₀ (13). The major exceptions are
526 the few genomic regions lacking USS. In NP these include the aforementioned 50 kb genomic
527 island and eleven 5-9 kb segments. The GG genome has no large segments without USS but has
528 twelve 5-9 kb segments and a 12 kb segment containing several integrases. Uptake ratios of 90%

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529 of the genome were within two-fold of the mean, which is consistent with previous analysis
530 showing that USS distributions are not strongly correlated with gene functions (23).

531 Uptake of DNA from other species can also influence recombination, either directly if the DNA is
532 sufficiently similar to *H. influenzae* DNA or indirectly if it competes for uptake of *H. influenzae*
533 DNA. Since the *H. influenzae* USS is shared with other Pasteurellacean species, both factors will
534 be important when *H. influenzae* shares the respiratory tract with coinfecting Pasteurellaceae.
535 Species that share the *Hin*-USS type of USS are expected to compete efficiently for uptake, with
536 recombination limited by sequence similarity, but uptake of DNA from species with the variant
537 *Apl*-USS type is known to be inefficient (22). Although this variant has the same inner core GCGG,
538 the first AT-tract and two outer core bases as the *Hin*-USS, these matches would only give an
539 average score of ~9.1 bits, too low for effective uptake by *H. influenzae*.

540 In the respiratory tract, the most important source of competing DNA is human cells. However,
541 our analysis suggests that *H. influenzae*'s uptake specificity allows its DNA to outcompete human
542 DNA, even if this is in 100-fold excess. This does not necessarily imply a selective advantage for
543 self-uptake, since USS accumulation in *H. influenzae*'s genome may simply be due to the
544 molecular drive process.

545 Uptake of DNA in the respiratory track could also be influenced by the presence of chromatin and
546 nucleoid proteins stably bound to the DNA. Although laboratory experiments typically use highly
547 purified DNA, cell death will release high concentrations of these proteins, which can contribute
548 significantly to biofilm stability (49). Because such proteins could interfere with uptake both
549 directly, by blocking binding to the USS, and indirectly, by blocking sliding of non-specifically
550 bound uptake machinery along the DNA, it will be important to reexamine DNA uptake using
551 DNA that retains its bound proteins.

552

553 **Methods**

554 **Bacterial strains, culturing, and competent cell preparations:** Growth and culturing of
555 *Haemophilus influenzae* strains that were used as donor (RR3133 and RR1361) and recipients
556 (RR3117 and RR3125) in the DNA uptake experiments followed standard methods (50).
557 Recipient strains RR3117 and RR3125 are both *rec2* derivatives of strain Rd KW20, with and
558 without a spectinomycin resistance gene respectively (Table S2). Donor strain RR3133 is an 86-
559 028NP derivative with a nalidixic acid resistance gene; and RR1361 is an unmodified PittGG
560 isolate. Strains were grown at 37 °C on brain-heart infusion broth supplemented with NAD
561 (2µg/ml) and hemin (10 µg/ml) (sBHI) with or without 1% agar to isolate single colonies on
562 plates or grow liquid cultures. To prepare naturally competent cells, cultures were maintained in
563 exponential growth for at least 2 hr, and at $OD_{600} = 0.2$, cells were collected by filtration from 10
564 ml of culture, transferred into starvation medium M-IV, and incubated at 37 °C for 100 minutes
565 before DNA uptake experiments (51).

566 **Input DNA preparations.** Donor DNA was purified using standard phenol:chloroform
567 extractions (52) from 10 ml overnight cultures of clinical strains 86-028NP and PittGG carrying
568 selectable markers (Table S2). High molecular weight DNA was then sheared into separate 'long
569 fragment' (1.5-9kb) and 'short fragment' (50-500bp) preparations using Covaris G-tubes and
570 sonication respectively. The fragment size distributions were measured using a Bioanalyzer with
571 the DNA 12000 kit (Agilent), dividing the relative fluorescence of each time point by its fragment
572 length estimated from the size standards. Fragment lengths were then grouped in classes of 10bp
573 for short fragments and of 200bp for large fragments. Using the large fragment distribution, in
574 our predictive model, grouped in 200bp bins took very long (an average of 184 seconds for

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575 100bp on a macOS Mojave v.10.14.5 with 8Gb of memory and a 2.2 GHz processor). For this
576 reason, we grouped fragment sizes in 1000bp bins, which reduced running times 12-fold. Results
577 with 1kb binds were nearly identical then when using 200bp bins.

578 **DNA uptake and recovery.** 10 ml of competent rec-2 mutant Rd cells in MIV were incubated
579 with 10 µg of sheared donor DNA for 20 min at 37 °C. To degrade remaining free DNA, the culture
580 was incubated with 1 ug/ml of DNase I for 5 minutes. Cells were washed twice by pelleting and
581 resuspension in cold MIV, and the final pellet was rinsed twice with cold MIV before
582 resuspension in 0.5 ml of extraction buffer (Tris-HCL 10mM ph 7.5, EDTA 10mM, CsCl 1.0 M).
583 Periplasmic DNA was extracted using the organic phenol:acetone extraction method as described
584 by (19,32,33) followed by an ethanol precipitation. DNA was resuspended by using 20 µl of
585 T10E10 buffer (Tris-HCl 10mM ph 7.5, EDTA 10mM). The DNA was then incubated at 37 °C with
586 400 ng of RNase A for 1 hour, followed by 30 min incubation with 30 ng of proteinase K to
587 remove RNase A. Recovered DNA was then separated from longer fragments of contaminating
588 genomic DNA by electrophoresis in a 0.8% agarose gel and recovered from the gel slice with a
589 Zymo gel DNA recovery kit. Recovered periplasmic DNA was quantified using both a Qubit dsDNA
590 HS Assay Kit (absolute DNA concentration) and by transformation into Rd (concentration of NaIR
591 donor DNA).

592 **DNA sequencing and data processing.** Sequencing libraries of the input and recovered DNA
593 samples were prepared using the Illumina Nextera XT DNA library prep kit according to
594 manufacturer recommendations. An Illumina NextSeq500 was used to collect 1-10
595 million paired-end reads of 2x150nt for each library (for >100-fold genomic coverage). Summary
596 statistics for each sample are provided in Table S2.

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597 *Reference sequences:* The original PittGG reference (NC_009567.1) generated by pyrosequencing
598 had many indel errors, so a new reference was constructed by Pacific Biosciences RSII of our
599 laboratory version of this strain (RR1361) (assembly by HGAP2 v2.0, followed by Circlator (53),
600 and then Quiver to polish the circular junction). Sequence references for this new PittGG
601 reference, as well as the genome references for 86-028NP (NC_007146.2) and Rd KW20
602 (NC_000907.1) were then corrected from input and control reads based on Illumina sequencing
603 using Pilon v1.22 (59). This was particularly important for the Rd KW20 recipient reference,
604 since the original (60) sequence dates from 1995 and contains several hundred ambiguous bases
605 and errors (10). This also accommodated differences between the sequence references and the
606 donor strains, which carried antibiotic resistance markers (Table S2).

607 *Chromosomal contamination measurements:* To identify and remove contaminating genomic
608 recipient reads in the recovered-DNA datasets, reads were aligned (via bwa mem v0.7.15,
609 samblaster v0.1.24, and sambamba v0.5.0) competitively to a concatenated reference sequence
610 consisting of the recipient Rd genome and the donor genome (NP or GG). Because the donor and
611 recipient genomes are distinguished by a high density of SNVs, as well as structural variation and
612 large indels (31,34,54), most contaminating Rd reads in uptake samples aligned to the Rd
613 reference while the desired periplasmic donor reads aligned to the donor reference. Reads that
614 mapped equally well to both genomes or to repetitive sequences within a genome were flagged
615 as low quality. The levels of uniquely aligned reads with quality > 0 that mapped to donor and
616 recipient chromosomes were used to calculate the percentage of contamination with recipient Rd
617 DNA (Table S2), and only the former were used for calculation of uptake ratios. Subsequent depth
618 of coverage values and summary statistics were extracted for all positions or specific intervals
619 using bedtools coverage v2.16.2 or sambamba flagstat (Table S2). All subsequent analyses and
620 plotting used the R statistical programming language, including standard add-on packages dplyr,

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621 tidy, plyr, ggplot2, data.table. Other packages used are specified below. Code is available at
622 https://github.com/mamora/DNA_uptake.

623 **Identifying USSs in the genomes.** Genomic USSs were identified by scoring each genome
624 position with the position-specific scoring matrix (PSSM) of Mell et al. (19); this is based on
625 uptake of synthetic fragments containing degenerate USS sequences. Positions scoring ≥ 10.0 or \geq
626 9.5 (maximum score is 12.6) were included in the standard (USS₁₀ and USS_{9.5}) lists of USS
627 locations. Since USS are asymmetric, USS positions in both orientations were specified by the
628 location of their central base 16. Sequence logos of USSs were generated using R package
629 seqLogo v. 3.8.

630 **Predicting DNA uptake from DNA sequence.** The predictive model is written in R v.3.5.1. Given
631 a list of USS positions and scores in a DNA genome of specified length, it uses a specified
632 distribution of DNA fragment sizes (over 10bp bins) to calculate the relative uptake of every
633 position in the genome. The genome is assumed to be circular. At each DNA position in turn, for
634 each 10bp bin of DNA fragment sizes, the model sums the predicted uptake contributions for
635 every fragment of that size that overlaps the position. For efficiency, the full calculation is only
636 done for the first position. At each subsequent position, the model calculates the new sum from
637 the previous position's sum by subtracting the contribution of the formerly leftmost fragment
638 and adding the contribution of the new rightmost fragment (Figure 2A).

639 Each fragment's contribution depends on the number of USS it contains, and on the scores and
640 separation of these USS. Fragments with no or incomplete USSs have baseline probabilities of
641 being bound (p_{bind}) and taken up (p_{uptake}); initial values for both = 0.02. For fragments with
642 one or more complete USS, p_{bind} depends on the fragment length (L) and on the separation of
643 the USSs if more than one is present, and p_{uptake} depends on the USS score(s). Initially p_{bind}

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644 for a fragment with one USS = $1 - L/17000$, assuming a maximum fragment length of 17kb. For a
645 fragment with 2 or more USS, the effective value of L was initially decreased by the separation
646 between the USS, so $p_{\text{bind}} = 1 - (L - \text{separ})/17000$. Initially p_{uptake} for a fragment with one
647 USS = $(\text{score} - 10)/\text{maxScore} - 10$, so p_{uptake} increased linearly from 0 for score = 10 to 1.0 for
648 score = 12.6. For a fragment with two or more USS, the mean of the USS scores was initially used.
649 After the experimental uptake ratios had been analyzed, both p_{bind} and p_{uptake} were modified
650 to use sigmoidal functions. The revised $p_{\text{bind}} = 1/(1 + \exp(7000 - L/-1500))$, where 7000bp is
651 the DNA length at the inflection point of the function and -1500 specifies the slope at this point.
652 The revised $p_{\text{uptake}} = 1/(1 + \exp(3.48(\text{score}-10.6))$, where 10.6 is the USS score at the inflection
653 point of the function and 3.84 is a value determining the slope at this point, estimated with the R
654 package Sicegar v. 0.2.2 (55), using USS scores and corresponding uptake ratios for a set of 209
655 USS_{9,5} isolated by at least 1000bp. A summary of each model parameters and equations is
656 included in Table S4.

657 Once the model has calculated the contributions of a specific fragment size to uptake of every
658 genome position, it moves on to the next size class. Once the contributions of every size class
659 have been calculated, the model combines all the contributions for each position, taking into
660 account the frequency of each size class in the input DNA. These position-specific uptake
661 predictions are then normalized to a mean uptake value of 1.

662 **Calculation of experimental uptake ratios from sequence coverage.** Uptake maps for each
663 donor DNA were created by dividing the mean of the three normalized recovered-DNA coverages
664 for each position by the corresponding normalized input-DNA coverage. Finally, uptake ratios
665 were normalized to a mean uptake of 1 over the entire genome and smoothed by calculating the
666 mean uptake over a 31bp central-oriented sliding window using function `rollapply` from R

667 package zoo v. 1.8-5. Because the replicates were extremely reproducible by Pearson correlation,
668 most plotting and analyses used the mean values.

669 **Periodicity analysis:** To detect possible periodic patterns in coverage depth and in uptake
670 ratios for the four datasets, periodograms were created using the R package TSA v. 1.2.

671 **Analysis of uptake ratio data:** To obtain a set of well-isolated USS₁₀s for analysis of peak
672 shapes, we identified the closest peak separation at which USS effects did not overlap by
673 examining sets of USS₁₀ that were separated by different distances (1200, 1000, 800, 600bp),
674 excluding positions with missing data and USS₁₀ that were 400bp or less from positions with low
675 input coverage (≤ 20 reads). Separation of ≥ 1000 bp was found to give the best compromise
676 between good peak separation and the number of USS₁₀s or USS_{9.5}s meeting the separation
677 criterion ($n=237$ and $n = 209$ respectively). To assess USS peak centrality and symmetry, we used
678 the sequences of 158 isolated USS₁₀s, that were at least 1000bp from the nearest USS_{9.5} and had
679 uptake ratios ≥ 3 . These sequences were aligned at position 16 of their USS after reverse-
680 complementing those with reverse-orientation USSs, and the mean and standard deviation of
681 uptake ratios at each position was calculated out to 100bp on either side of the USS. Differences
682 between the left and right sides were assessed with a Student's t-test ($P > 0.05$).

683 **Contributions of weak USSs:** To look for weak uptake effects in the valleys between USS-
684 associated uptake-ratio peaks, a 'far from USS₁₀' subset of NP positions was created, consisting of
685 positions that were at least 0.6kb from the closest USS₁₀. This gave 575 segments summing to
686 29% of the genome. Each of these regions was searched for positions with uptake ratios > 0.2 .
687 Uptake maps containing positions with uptake ratios > 0.2 were plotted, including flanking
688 positions out to 2kb, to identify effects of USS with scores < 10 . Uptake ratios at all the USS_{9.5-10}s
689 in the 'far from USS₁₀' dataset ($n=99$) were then examined. Mean uptake of each USS_{9.5-10} was

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690 calculated and a boxplot was built grouping USS_{9.5-10s} by score. Significance of differences
691 between the score groups was evaluated using a t-test. A two-proportions power analysis was
692 used to measure the effect that could be detected with the current number of USS_{9.5-9.6} and
693 USS_{9.6-9.7}, using R package pwr v. 1.2-2.

694 **Incorporating within-USS interaction effects into uptake predictions:** Figure 6 of Mell et al.
695 (19) shows the strength and direction of pairwise interaction effects between USS positions.
696 From this figure we extracted the mid-range value of the interaction effect at each interacting
697 pair of USS positions (only some pairs of positions showed such effects). For each NP USS_{9.5}
698 whose sequence differed from the USS consensus at both positions of such a pair, the USS score
699 was modified by adding or subtracting the corresponding interaction value. The modified scores
700 were then used by the model to predict DNA uptake, as described above.

701 **Simulated noise analysis:** Noise-free uptake data for short and long fragments was simulated
702 by raw input coverage data for NP-short (sample UP07) and NP-long (sample UP03) that had
703 been smoothed using a LOESS regression and normalized to a mean coverage of 1.0. Amplitude of
704 three types of noise ('white', 'pink', and 'red') were generated for every genome position using
705 the 'tuneR' R-package (56).

706 To determine the level of noise to be added, for each genomic position, we first calculated the
707 difference in normalized coverage (depth per million reads) of each replicate from the mean of
708 the three replicates, grouping the normalized coverage-differences by the normalized mean
709 coverage at the 3 replicates that was used in the subtraction before. Next, we calculated the
710 maximum coverage-differences according to each mean normalized coverage value. This number
711 was multiplied by the simulated amplitude of red noise and by 1, 1.5, 2, 2.5 or 3 to estimate the
712 level of noise to be added to each position according to its coverage. The most appropriate type

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713 of noise was identified by examining the autocorrelations of simulated coverages after noise was
714 added. Adding red noise to each position at levels proportional to the observed coverage-
715 dependent variation gave an autocorrelation of 0.999, identical to that of the real data.

716 **Data availability:** All short read data have been deposited at NCBI under BioProject
717 PRJNA387591 and BioSamples are listed in Table S2. The PacBio-sequenced PittGG genome
718 reference was deposited into Genbank under SRA number SRR10207558. Full calculations,
719 processed datasets, and Rscripts available at: https://github.com/mamora/DNA_uptake.

720 **Ethics approval and consent to participate**

721 Not applicable

722 **Consent for publication**

723 Not applicable

724 **Competing interests**

725 The authors declare that they have no competing interests

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729

730 **Authors' contributions**

731 RR and JCM conceived the study. MM and RR wrote the manuscript and performed the bioinformatic
732 analysis. MM did the DNA uptake experiments. JCM did the library preparation, sequencing and
733 sequence alignments. GE and RE did the genome assembly of the PittGG genome.

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870 **Figure legends**

871 **Figure 1. A.** USS sequence logo based on the DNA-uptake position weight matrix from Mell et al.

872 (19) uptake bias sequence logo. **B.** Conserved USS segments

873 **Figure 2. A.** Components of the DNA uptake model (see Methods for details). **B. & C.** Model I

874 predictions for uptake centered at a 12 bit USS for: **B.** 100, 200, and 300 bp fragments, **C.** a mixed

875 distribution of fragments between 25-300 bp with and without baseline uptake. **D. E. & F.** Model

876 predictions for uptake of a 3000 bp region with 3 USSs (black squares, scores in red) using

877 different fragment-length distributions: **D.** 50-300 bp fragments, **E.** 50-2000 bp fragment, **F.** 1-14

878 kb fragments. **G. & H.** Predicted DNA uptake of a 50 kb segment using different fragment-length

879 distributions: **G.** NP-short fragment length distribution, **H.** NP-long fragment length distribution.

880 **I.** Locations and scores of USS_{10s} in this 50 kb segment.

881 **Figure 3.** Local uptake ratios (smoothed over 31 bp) for the same 50 kb segment of the NP

882 genome as Fig. 2G & H. Grey points indicate positions with input coverage lower than 20 reads.

883 Gaps indicate unmappable positions. **A.** Uptake ratios of short-fragment DNA. **Inset:** Same data

884 with a logarithmic-scale Y-axis. **B.** Uptake ratios of long-fragment DNA. **C.** Locations and scores

885 of USS_{10s}.

886 **Figure 4:** Predicted and observed DNA uptake analysis for different model versions. **A.** and **C.**

887 Blue lines show the same uptake ratio maps as in Fig. 3A. **A.** Orange line shows the same

888 predicted uptake as in Fig. 2G, using model I settings (baseline binding and uptake $p=0.2$, linear

889 uptake function). **C.** Orange line shows predicted uptake using model II settings (baseline binding
890 and uptake $p=0.02$, sigmoidal uptake function). **B.** Relationship between USS score and uptake
891 ratio peak height in NP-short dataset for isolated USS_{9.5}s (black points, N= 209 USSs separated by
892 at least 1000 bp), and uptake functions used to predict uptake. **Blue line**, linear uptake function
893 used in the model I; **orange line**, sigmoidal uptake function used in the model II.

894 **Figure 5.** Predicted shape features of USS with strong and weak peaks. Thick grey lines: shape
895 analysis of consensus USS sequence. Blue and orange lines: shape analysis of genomic USS
896 separated by at least 500 bp, grouped by uptake ratio. **A-D:** USS_{10.0-10.5}. Blue: USS with weak
897 peaks (uptake ratios <0.6 , $n=47$, mean score=10.22). Orange: USS with strong peaks (uptake
898 ratios >2.0 , $n=10$, mean score=10.26). **E-H:** USS_{10.5-11.0}. Blue: USS with weak peaks (uptake ratios
899 <0.6 , $n=14$, mean score=10.64). Orange: USS with strong peaks (uptake ratios >2.0 , $n=59$, mean
900 score=10.79). **I-L** DNA shape of the USS_{9.5-10} with uptake higher (red, $n = 10$) and lower (blue $n =$
901 68) than 0.2. **A, E and I.** Minor groove width, in Å. **B, F and J.** Propeller twist, in degrees. **C, G and**
902 **K.** Helix twist, in degrees. **D, G and L.** Base pair roll, in degrees. Coloured bars indicate
903 components of the USS (see Figure 1): light orange, outer core; dark orange, inner core; green, AT
904 tracts.

905 Supplementary information

906 **Additional file 1: Supp. Figure 1.** Frequency distribution of USS scores for all positions in the
907 NP, GG, and Rd genomes and for four random-sequence genomes with the same base
908 composition. **Supp. Figure 2:** Distributions of DNA fragment lengths. **Supp. Figure 3.** Local
909 uptake ratio maps. **Supp. Figure 4.** Sources of variation in read coverage. Read coverage maps
910 for NP long-fragment samples over a 50 kb segment of the genome. **Supp. Figure 5.** Tests of
911 periodicity by Fourier-transform analyses performed with R-package RCA. **Supp. Figure 6.**
912 Frequencies of uptake ratios below 0.1. **Supp. Figure 7.** Symmetry and centrality of uptake
913 peaks. **Supp. Figure 8.** Effects of interactions between USS positions on USS scores. **Supp.**
914 **Figure 9.** Uptake ratios at weak USSs. **Supp. Figure 10.** Analysis of DNA uptake effects of USS₁₀
915 pairs. **Supp. Figure 11.** Predicted and observed uptake of long NP DNA fragments. **Supp. Figure**
916 **12:** Distributions of long fragment input (blue) and recovered (purple) DNA fragment lengths.
917 **Supp. Figure 13.** Uptake of long NP DNA fragments as a function of local USS₁₀ density. **Supp.**

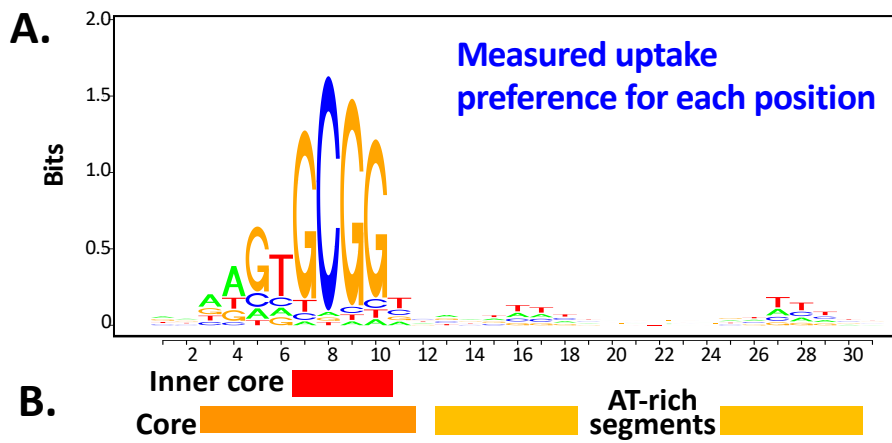
918 **Figure 14.** Correlation coefficient between simulated signal with and without different levels of
919 noise. **Supp. Figure 15.** Predicted and observed uptake of GG short and long fragments. **Supp.**
920 **Figure 16.** Uptake ratio of NP and GG positions with input coverage higher or lower than 20
921 reads

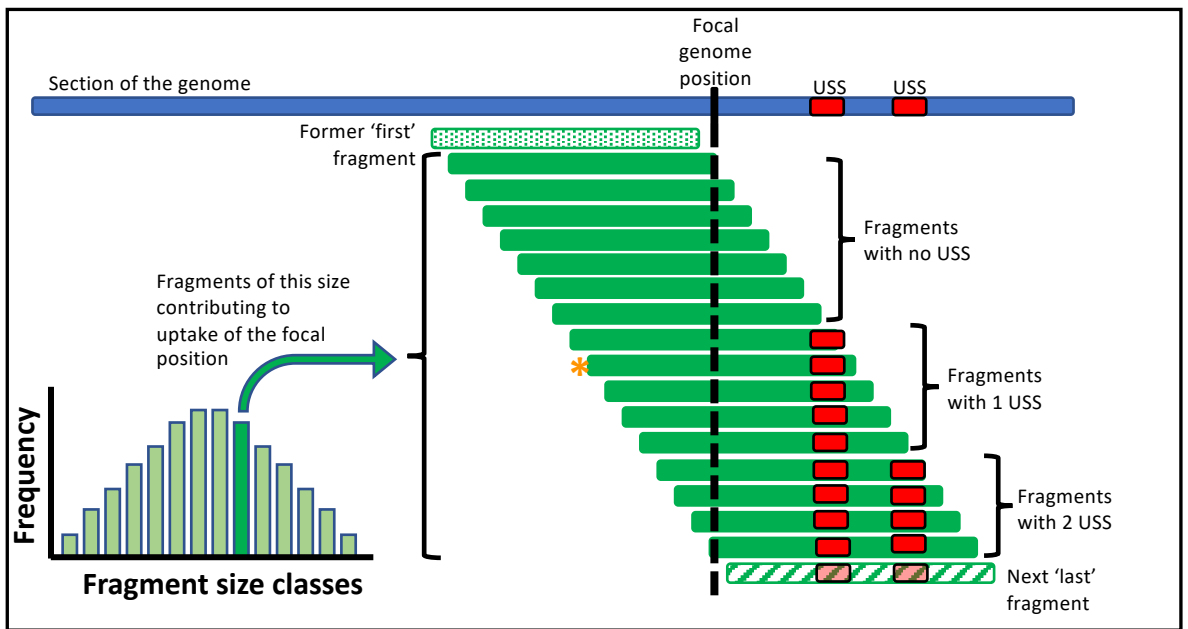
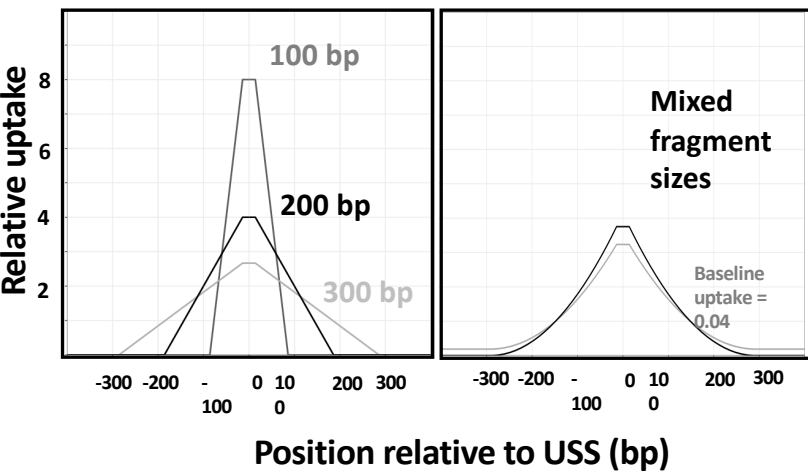
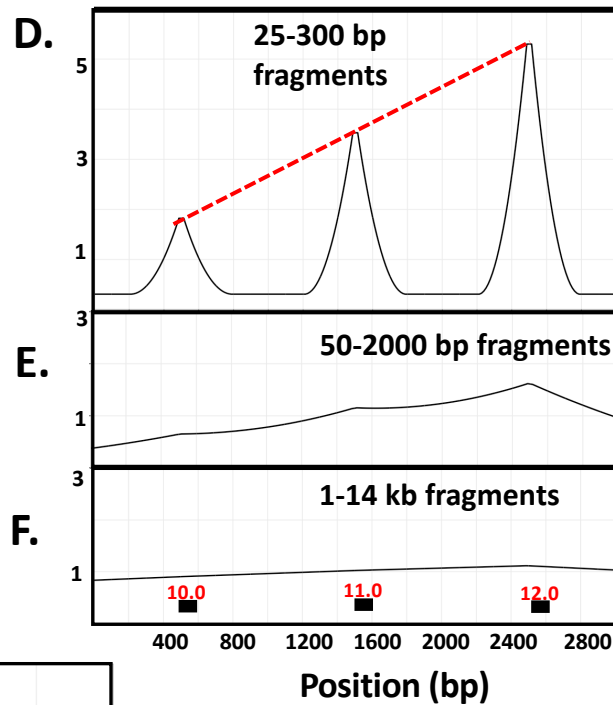
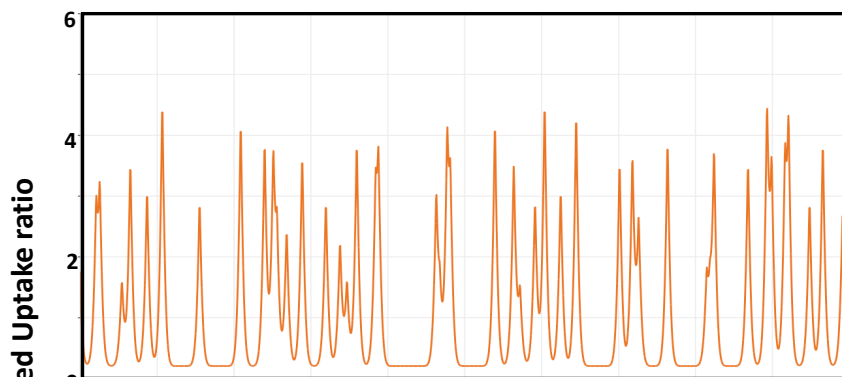
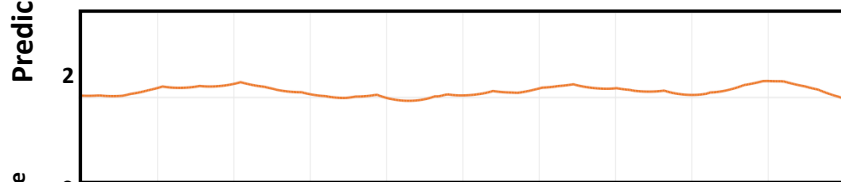
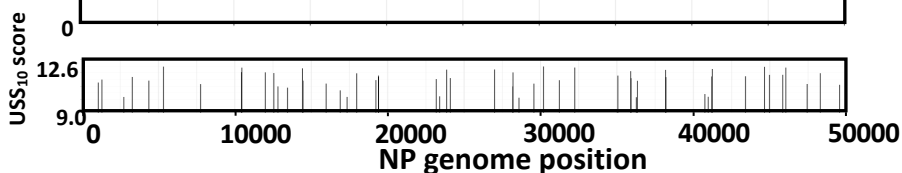
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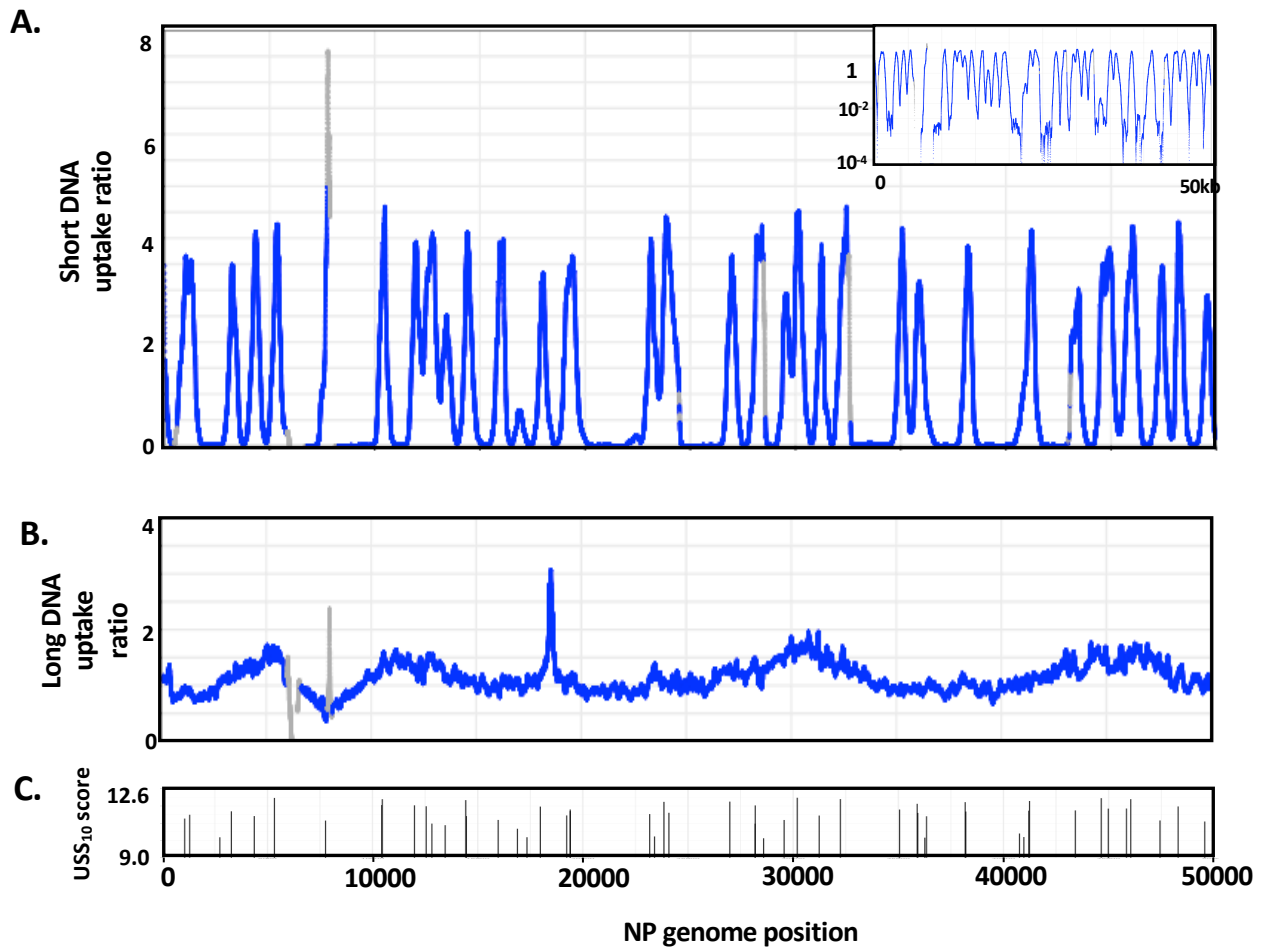
923 **Additional file 2: TableS1** uptake-prediction position-specific scoring matrix from Mell *et*
924 *al.*'s degenerate-sequence uptake experiment. **Table S2** Detailed sequencing information about
925 all samples. **Table S3** Proportion of positions in NP and GG with high and low uptake that are
926 biased towards low input coverages. **Table S4** Summary of the three models and their
927 parameters. **Table S5** Relative simulated uptake of human and NP 1kb and 10kb DNA fragments.

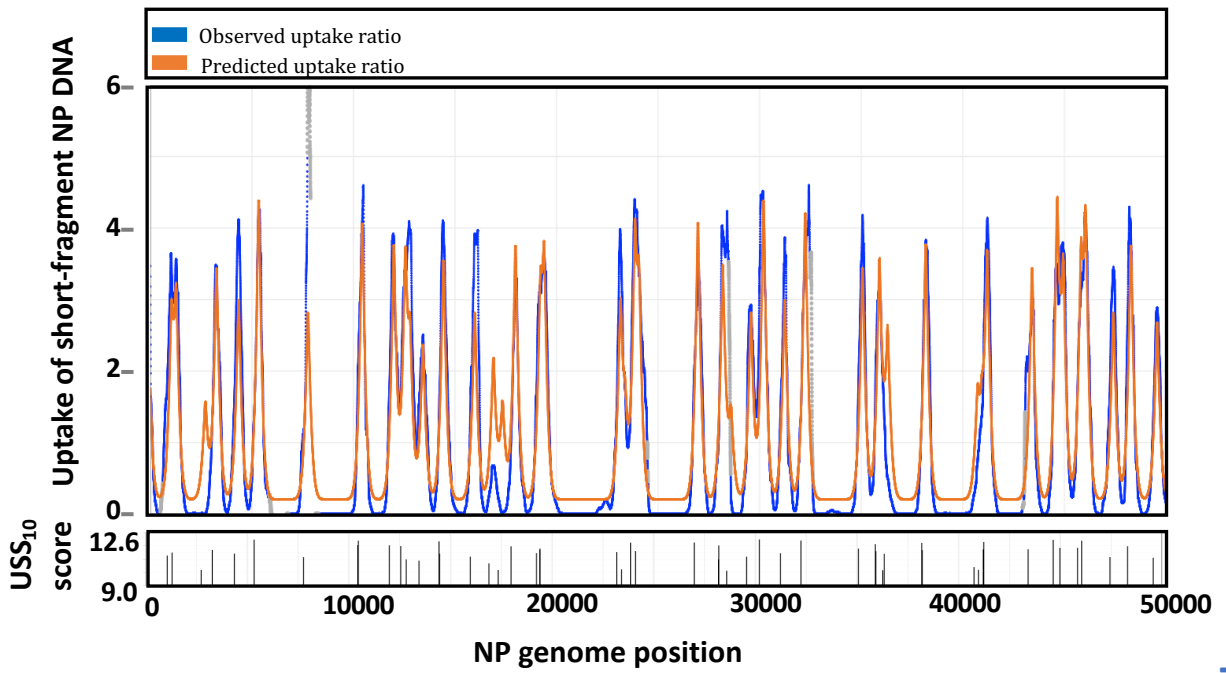
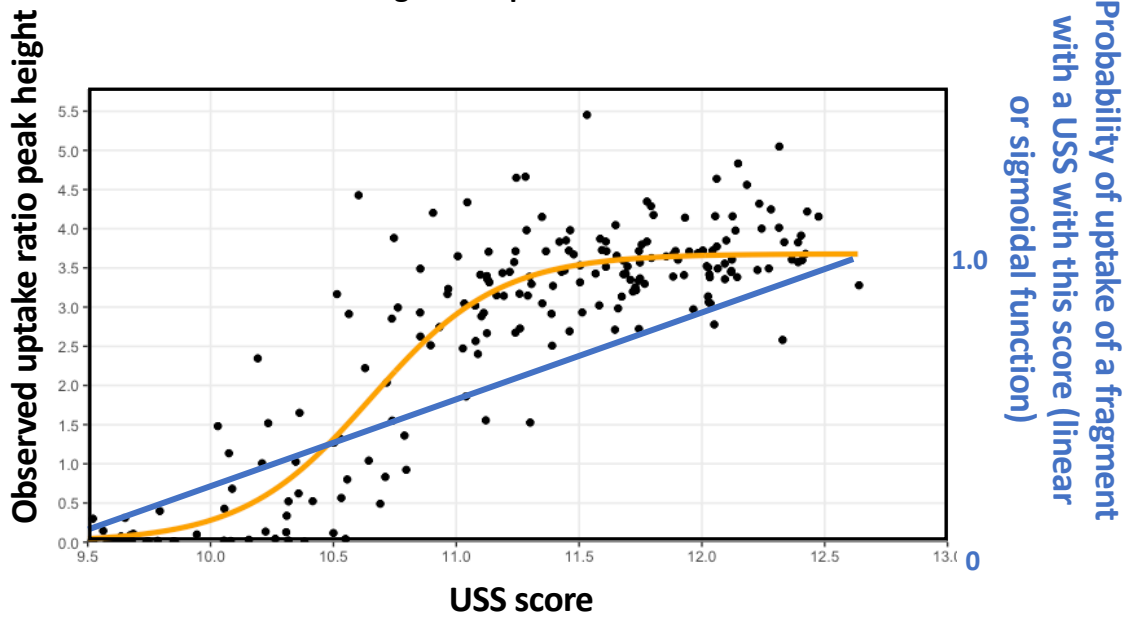
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A.**B.****D.****G.****H.****I.**



A.**B.****C.**