

Natural conjugative plasmids induce bacterial biofilm development

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Horizontal gene transfer is a principal source of evolution leading to change in the ecological character of bacterial species¹. Bacterial conjugation², which promotes the horizontal transfer of genetic material between donor and recipient cells by physical contact, is a phenomenon of fundamental evolutionary consequence³. Although conjugation has been studied primarily in liquid, most natural bacterial populations are found associated with environmental surfaces in complex multispecies communities called biofilms⁴. Biofilms are ideally suited to the exchange of genetic material of various origins, and it has been shown that bacterial conjugation occurs within biofilms^{5,6}. Here I investigate the direct contribution of conjugative plasmids themselves to the capacity of the bacterial host to form a biofilm. Natural conjugative plasmids expressed factors that induced planktonic bacteria to form or enter biofilm communities, which favour the infectious transfer of the plasmid. This general connection between conjugation and biofilms suggests that medically relevant plasmid-bearing strains are more likely to form a biofilm. This may influence both the chances of biofilm-related infection risks and of conjugational spread of virulence factors.

I used high-debit, continuous flow cultures to monitor biofilm formation of various *Escherichia coli* K12 laboratory strains on submerged, removable Pyrex slides. Strains carrying the F episome,

such as *E. coli* TG1, formed a thick biofilm in 24 h (up to 500 μm with 2×10^{10} colony-forming units (CFU) per cm^2) (Fig. 1a; see also Supplementary Information Fig. 4). In contrast, after 24 h all the F⁻ strains tested only formed microcolonies whose development was not sufficient to be observed macroscopically (8×10^5 CFU cm^{-2} ; Fig. 1a, b). To demonstrate that this phenotype was due to the F factor, I removed the plasmid from TG1 and consequently TG1 F⁻ lost its ability to form a biofilm (Fig. 1a). Furthermore, when conjugated into MG1655, the F plasmid promoted biofilm formation in this and all F⁻ strains tested (Fig. 1c).

The physical nature of conjugation (cell-cell contact through specialized conjugative pili), along with the observation that various pili-like cell appendages are involved in biofilm formation^{7,8}, led me to test the role of conjugative pili produced in *E. coli* carrying the F factor in this biofilm process. I generated a non-polar mutation of the gene that encodes the pilin subunit of the conjugative pilus, *traA*, located within the large *tra* operon⁹ (Fig. 2a). Strain TG1 *traA::aphA* did not form a biofilm and this capacity is restored by complementation with a plasmid bearing the wild-type *traA* allele (Fig. 2b). The same observations were made using transfer-deficient F plasmids indicating that the phenomenon does not require conjugative DNA transfer itself. This result demonstrated the role of the conjugative F pilus in biofilm formation.

The kinetics of surface adhesion in the F⁻ and F⁺ *E. coli* strains MG1655 and MG1655 F, respectively, showed that expression of the F pilus accelerated both the initial adhesion events and biofilm development (Fig. 1b; see also Supplementary Information Fig. 5). In the culture conditions, biofilm development does not result from recruitment of planktonic cells but from internal growth on the available surfaces. To determine whether pilus-specific donor-recipient interactions could mediate cell contacts, I tested the effect of a mutation in the gene coding for the outer membrane protein OmpA on biofilm growth. OmpA reduces F pilus-specific

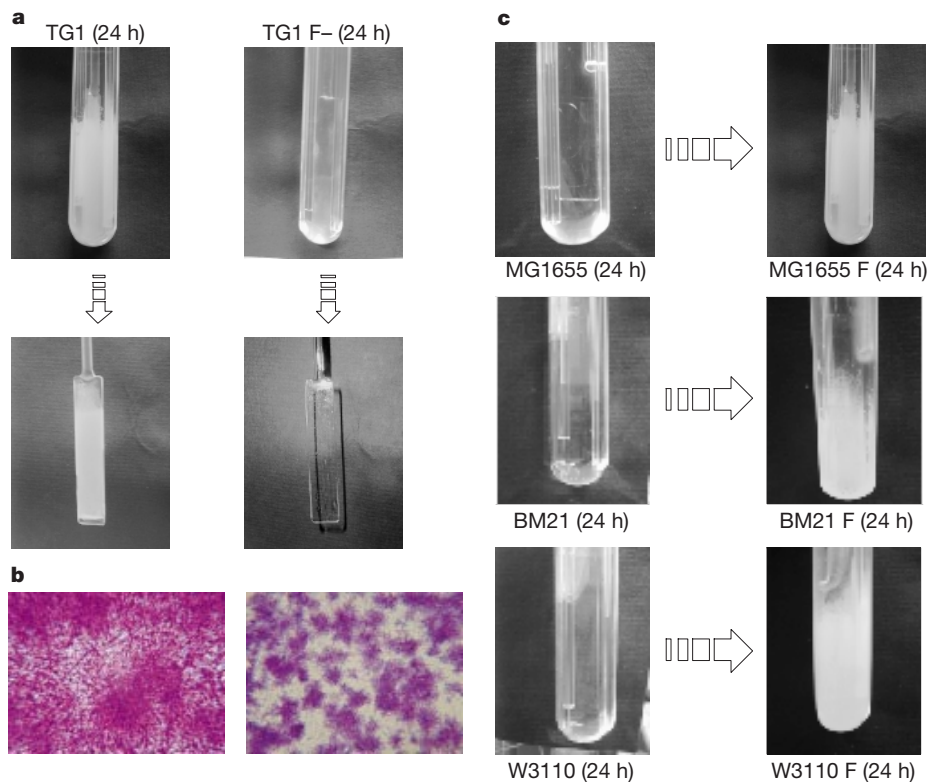


Figure 1 The F episome induces the formation of a thick biofilm in *E. coli*. **a**, Biofilm formation in TG1 and TG1 F⁻ strain. Top, biofilm phenotype in microfermenters. Bottom, biofilm phenotype on the corresponding Pyrex slides removed from the microfermenters. **b**, Comparison of cell density of strain MG1655 F and MG1655 after 24 h of culture in

microfermenters. Transmitted light microscopy of the Pyrex slide stained with crystal violet. Left, MG1655 F biofilm in the area that was least dense. Right, MG1655 biofilm in the area that was most dense. Scale bar, 10 μm . **c**, Biofilm formation in three different *E. coli* K12 strains. Left, F⁻ strains; right, F⁺ strains.

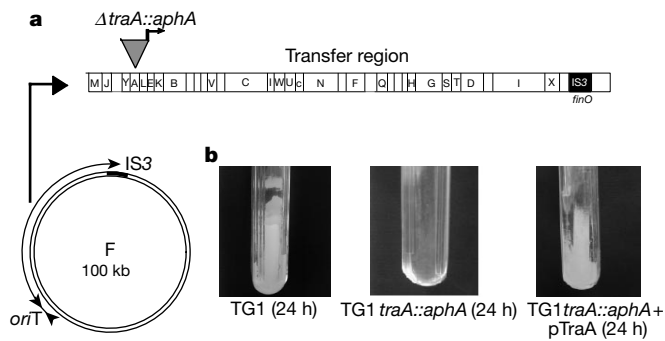


Figure 2 F pilus synthesis is required for biofilm formation in TG1. **a**, Schematic genetic map of the F plasmid's *tra* operon (33.3 kilobases). **b**, Biofilm formation by TG1 after 24 h (left). Biofilm formation by TG1 *traA::aphA* after 24 h (middle). Biofilm formation by TG1 *traA::aphA* complemented with plasmid pTraA after 24 h (right).

contacts with the recipient cell during conjugation¹⁰. No reduction in the extent of biofilm growth could be observed in an F+ *ompA* mutant compared to its wild-type parental isogenic strain. These results suggest that the F conjugative pilus acts as an adhesion factor that allows non-specific cell–surface or cell–cell contacts leading to the observed three-dimensional growth of the biofilm.

I proposed that plasmids isolated from natural populations of Gram-negative bacteria could also promote biofilm formation. Twenty-nine natural conjugative plasmids were tested for their capacity to promote biofilm formation. Out of these 29 plasmids nine from six different incompatibility groups (Table 1; see also

Supplementary Information Fig. 6) led to a biofilm. This demonstrated that induction of biofilm formation is not restricted to a specific incompatibility group nor to a specific type of pili.

These results also showed that plasmids known to be constitutive for pilus synthesis allowed the formation of a biofilm whereas most of the plasmids described as repressed did not (Table 1). Repression results in very few bacteria (0.1%) with conjugative ability, which probably minimizes the burden on the host arising either from the energy expended to maintain the conjugative apparatus or from associated properties, such as pili-specific bacteriophage sensitivity. I investigated the relationship between the regulation of pilus synthesis and the ability to form a biofilm, and compared the repressed F-like natural plasmid, R1, with its permanently derepressed mutant R1-*drd19*. Although MG1655 carrying R1 did not form a biofilm, its mutant R1-*drd19* promoted the formation of a thick biofilm (Fig. 3a). During the conjugative process, chance encounters between rare transfer-proficient bacteria that carry a repressed plasmid and potential recipients initiate a cascade of conjugative transfer because newly transferred episomes are transiently derepressed. This phenomenon, called ‘epidemic spread’, proceeds until all potential recipients have acquired the plasmid¹¹. To further investigate how transitory derepression of the conjugative apparatus could lead to the development of a biofilm, I co-inoculated the donor *E. coli* strain MG1655 R1 with the recipient BM21. This led to an intermediate phenotype between the biofilm capacity of MG1655 R1 alone and MG1655 R1-*drd19* (Fig. 3b). This suggested that the transient derepression of the R1 conjugative functions due to contact with the recipient cells can promote biofilm formation. Such a situation is likely to occur when planktonic bacteria carrying plasmids come into proximity with a biofilm

Table 1 Biofilm formation promoted by natural conjugative plasmids

Strain	Original host†	Type of pili‡	Pilus synthesis‡	Biofilm per se	Biofilm after co-inoculation	
TG1	NA	Thick flexible	Constitutive	+	NA	
MG1655	NA	none	NA	–	NA	
BM21	NA	none	NA	–	NA	
W3110	NA	none	NA	–	NA	
Incompatibility group*	Plasmid‡	Original host‡	Type of pili‡	Pilus synthesis	Biofilm per se	Biofilm after co-inoculation
IncFI	F'lac	<i>Escherichia coli</i> K12	Thick flexible	Constitutive	+	ND
	PIP162	<i>Salmonella enterica</i> ser. typhi	Thick flexible	Repressed	–	+
	RIP162-2	<i>Salmonella enterica</i> ser. typhi	Thick flexible	Repressed	–	+
IncFII	R1	<i>Salmonella enterica</i> ser. paratyphi	Thick flexible	Repressed	–	+
	R1 <i>drd19</i>	–	–	Constitutive	+	ND
	R1-16	–	–	Repressed	–	+
	PIP24	<i>Shigella sonnei</i>	–	Repressed	–	+
IncI1	pIP112	<i>Salmonella enterica</i> ser. panama	Thin flexible	Repressed	–	+
	R64	<i>Salmonella typhimurium</i>	–	Repressed	–	+
IncJ	R391	<i>Providencia rettgeri</i>	Thick flexible	Repressed	–	+
IncI2	TP114	<i>Escherichia coli</i>	Thin flexible	Repressed	+	ND
	PIP175	<i>Salmonella enterica</i> ser. Wien	Thin flexible	Repressed	–	+
Inc9	RIP71a	<i>Escherichia coli</i>	Thick flexible	Repressed	–	+/-
IncH2	TP116	<i>Salmonella enterica</i> ser. typhi	Thick flexible	Repressed	+	ND
IncX	PIP1100	<i>Escherichia coli</i>	Thick flexible	Repressed	+/-	+
	R6K	<i>Providencia rettgeri</i>	Thick flexible	Repressed	–	+
Inc10	PIP72	<i>Escherichia coli</i>	Thick flexible	Repressed	–	+
IncA/C	R16a	<i>Providencia stuartii</i>	Thick flexible	Repressed	–	+
	pIP55	<i>Klebsiella pneumoniae</i>	Thick flexible	Repressed	+/-	+/-
IncT	pRtsI	<i>Proteus vulgaris</i>	Thick flexible	Repressed	+/-	+
Inc7/L/M	pIP135	<i>Enterobacter cloacae</i>	Rigid	Repressed	+	ND
	pIP69	<i>Salmonella enterica</i> ser. paratyphi	Rigid	Repressed	–	+/-
	R69-2	–	Rigid	Repressed	+	ND
	pIP113	<i>Salmonella enterica</i> ser. panama	Rigid	Repressed	+	ND
IncP	RN3	<i>Shigella</i> sp.	Rigid	Constitutive	+	ND
	RP4	<i>Pseudomonas aeruginosa</i>	Rigid	Repressed	–	+
	RP1	<i>Pseudomonas aeruginosa</i>	Rigid	Repressed	–	+
IncW	R702	<i>Proteus mirabilis</i>	Rigid	Repressed	–	+
	RSa	<i>Shigella</i> sp.	Rigid	Constitutive	+	ND
	R388	<i>Escherichia coli</i>	Rigid	Constitutive	+	ND

NA, not applicable; ND, not determined; +, formation of a thick biofilm (2.1×10^8 CFU cm⁻²); +/-, formation of a weak but visible biofilm (5.1×10^7 CFU cm⁻²); –, no biofilm macroscopically observed ($<10^5$ to 10^6 CFU cm⁻²).

* Data from ref. 16.

† Data from ref. 17.

‡ Data from refs. 18 and 19.

population composed of potential recipient cells, that is, bacteria that do not carry conjugative plasmids of the same incompatibility group.

To mimic a situation where donor bacteria carrying repressed conjugative plasmid(s) would encounter a surface-attached recipient population, I first inoculated BM21 for 24 h. These bacteria reached the surface and formed microcolonies without much further development. After 24 h, the co-inoculation with MG1655 R1 led to the formation of a biofilm similar in extent to the one formed by MG1655 R1-*drd19*, where up to 90% of the cells corresponded to recipient bacteria that received R1, less than 2% to donor bacteria (MG1655 R1) and up to 10% of plasmid-free, initial recipient cells (BM21). This suggests that derepression leads to both biofilm formation and horizontal transfer of the plasmid to the recipient cells. In a reverse experiment where the donor MG1655 R1 was inoculated 24 h before the recipient BM21, both the extent of transfer and biofilm development were reduced (Fig. 3b). This may be a consequence of issues concerning accessibility, where surface-attached MG1655 R1 are not in a position to interact efficiently with a high enough number of planktonic recipient cells.

I performed co-inoculation experiments with the 20 strains carrying plasmids that do not promote a thick biofilm formation on their own (Table 1). When the plasmid-free MG1655 strain was inoculated 24 h before the plasmid-carrying strains, 17 out of 20 of these strains produced a thick biofilm (Table 1; see also Supplementary Information Fig. 7). Thus, 90% (26) of the 29 natural conjugative plasmids that were tested induced the development of a biofilm either alone or in the presence of recipient cells.

I propose that biofilm formation proceeds through the introduction of a small sub-population of donor bacteria expressing the conjugative pili into the surface-attached recipient population. After this initial event, the conjugative transfer of the plasmid occurs and the conjugative pili synthesis is derepressed in the transconjugants and their immediate descendants. The recipient population transiently expressing conjugative pili would then form a biofilm through pilus-mediated networking within the fast-growing upper layer bacteria of the biofilm (see Fig. 3c). On completion of the plasmid transfer to the surface-attached bacteria, the conjugative pili would recede and be replaced by adhesion

factors (such as other types of pili or exopolysaccharides), maintaining the coherence of the biofilm.

I have shown here that conjugative plasmids express factors that favour the access of planktonic bacteria to biofilm communities. By doing so, they increase their chances of being transferred to a large number of recipient cells, and support the emerging hypothesis that biofilms may constitute ecological niches that are highly favourable to conjugation¹².

The persistence of nearly ubiquitous plasmids in natural bacterial populations requires one or both of two basic mechanisms: parasitic transmission through horizontal transfer or vertical transmission through maintenance in the host progeny. Empirical studies and mathematical models suggest that plasmids cannot persist solely by carrying genes that are beneficial to their bacterial host¹³. On the other hand, it has been proposed that plasmids cannot be maintained as pure genetic parasites owing to their low rate of infectious transmission, which cannot overcome the counter selection due to the burden that their replication imposes on their host. The transmission of bacterial plasmids has therefore often been considered to be a trade off between horizontal and vertical modes of transmission. However, these assumptions were made on the basis of a limited number of laboratory studies essentially performed in liquid culture, where transfer dynamics may not reflect those of natural bacterial populations such as biofilms¹³.

I propose that conjugative plasmids favour the incorporation of their host into biofilm environments where the density of potential bacterial recipients is sufficient for plasmids to transfer at high rates and to be maintained by infectious transmission alone within some natural habitats. Hence, the expression of biofilm-promoting factors, without having an *a priori* benefit for their host, could sufficiently enhance the probability of the infectious transfer rate so as to offset the segregation loss.

Joining a biofilm community may also have numerous beneficial aspects for the bacterial host itself, such as collective defence against physical and chemical environmental stresses¹⁴. Such ecologically beneficial factors expressed by plasmids may provide a rationale for the unexplained vertical maintenance of the numerous uninfected cryptic plasmids found in natural bacterial populations.

Conjugative plasmids mediate the horizontal transfer of anti-

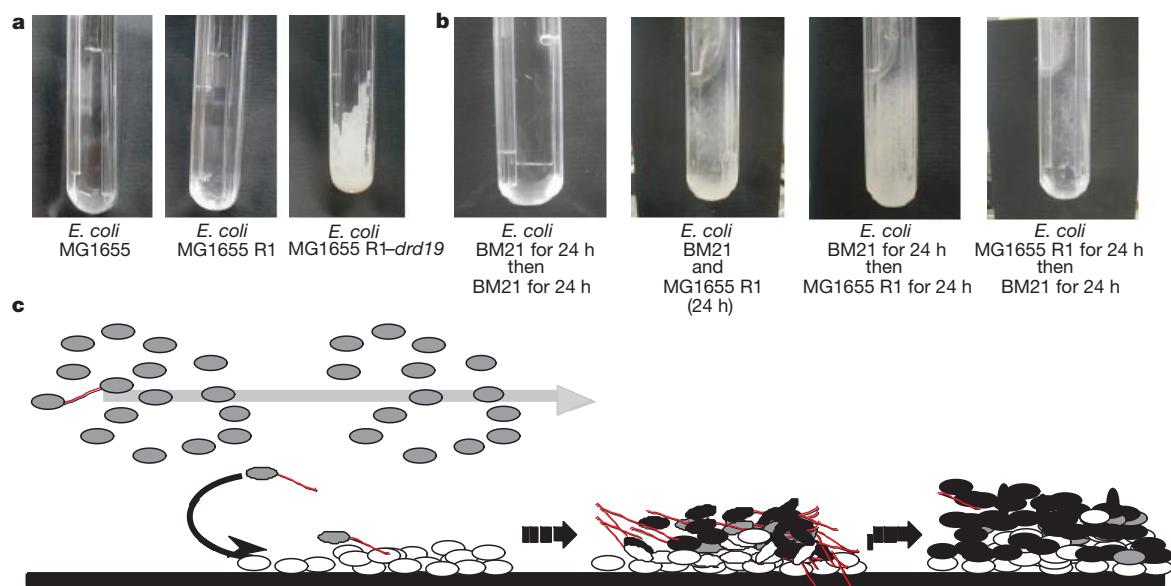


Figure 3 Derepression of conjugation ability induces biofilm development. **a**, Biofilm formation comparison between plasmid-free MG1655 strain (left), and MG1655 carrying the repressed conjugative plasmid R1 (middle) or its derepressed mutant R1-*drd19* (right). **b**, Co-inoculation experiments. **c**, Schematic model. Grey planktonic cells indicate

donor cells carrying the conjugative plasmids, which express a biofilm-promoting factor (for example, conjugative pilus in red). White cells indicate recipient cells and black cells indicate transconjugants (see text).

biotic resistance and virulence factors. The finding of a connection between bacterial conjugation and biofilm formation suggests that an important ecological consequence of the use of antibiotics and biocides in clinical medicine and agriculture may have been the selection of plasmid-bearing strains that are more likely to form a biofilm. Because biofilms are a common cause of persistent nosocomial infections that are difficult to eradicate owing to innate physiological properties¹⁴, this aspect may prove to be of relevant medical significance in addition to the conjugational spread of virulence factors themselves. □

Methods

Bacterial strains and plasmids

Bacterial strains are listed in Table 1 and were provided by the Collection of the Institut Pasteur (<http://www.pasteur.fr/applications/CIP/>) and the Unité des Agents Antibactériens (P. Courvalin and G. Gerbaud).

Biofilm

All experiments were performed in triplicate in 0.4% glucose M63B1 minimal medium at 37 °C. Continuous 60-ml microfermenters with four liquid and gas sampling ports (Pasteur Institute's Laboratory of Fermentation) were configured as continuous-flow culture bioreactors with a 40 ml h⁻¹ flow rate (F). 10⁸ bacterial inocula from overnight precultures grown in glucose minimal medium with required antibiotics were used to inoculate microfermenters, which were then cultivated for 3–48 h. The culture volume (V) was constant and the imposed dilution rate (D) was $D = F/V = 0.66 \text{ h}^{-1}$. Hence, the theoretical generation time (T) required for constant density culture in the micro-fermenter was $T = \ln 2/D = 1.05 \text{ h}$. The average generation time calculated in exponential batch culture for *E. coli* strains MG1655 and BM21 was 1.3 h. Therefore, the high input rate of fresh, diluting medium used in our experimental model was imposed to avoid any significant planktonic growth. Stirring was assured by aeration with sterile pressed air (0.3 bar). Submerged, removable Pyrex slides (total area of 22.4 cm²) served as growth substratum.

Microscopy and image analysis

Biofilm development was recorded with a Nikon Coolpix 950 digital camera. Epifluorescence, phase contrast and transmitted light microscopy were acquired with a Leitz Dialux 20EB microscope equipped with ×25 to ×100 objectives. Scanning confocal microscopy was performed at the confocal microscopy station of the Pasteur Institute.

Non-polar deletion of the *traA* gene

A non-polar mutation deleting the entire *traA* gene was created by allelic exchange¹⁵ using the primers TraAGB-5: 5'-AGGGAGGCAGATAAAGAGGAAGATATAACATTTAATACA CTCTAGTTTATTCATTTATCCGAAATTGAGGTAACCTATGAAAGCCACGGTTGTG TCTCAA-3' and TraAGBnp-3: 5'-CGCGCTCTGGTTGGTCAGTGTTCGGGAAACG ATATTTCTTAAGTTTATTCCTCGTCTCCGACATCGTTTATTTCTCTGTAGAAAAA CTCATCGAGCA-3', and *aphA* gene (kanamycin resistance) from Tn903 as template. The mutation was verified by PCR analysis.

Cloning of the *traA* gene

pTraA was constructed by PCR amplification of *traA* from strain TG1 using the primers TraAecorbs-5: 5'-AAAGAATTCGAAATGAGGTAACCTATGAATGC-3' and TraAH3-3: 5'-CCCAAGCTCTCGTTTATTTCTCTGTCAGAG-3'. I verified the nucleotide sequence of the construction.

Biofilm co-inoculation procedures

A preculture of a recipient strain BM21 (nal^r (nalidixic acid resistant)) was inoculated for 24 h and then co-inoculated with MG1655-S R1 (St^r (streptomycin resistant), Ap^r (ampicillin resistant) and Km^r (kanamycin resistant)) for another 24 h. Pyrex slides were removed and centrifuged in 15 ml of fresh M63B1 medium for 1 min. The number of colony-forming units was determined by plating serial dilutions of the resuspensions on medium supplemented with nalidixic acid (for recipient BM21 scoring), streptomycin, ampicillin, kanamycin (for donor MG1655-S R1 scoring), nalidixic acid, ampicillin and kanamycin (for BM21 R1 transconjugant scoring), and without antibiotic (for total cell scoring). Co-inoculation with BM21 strains carrying the plasmids described in Table 1 were generated using MG1655-S as recipient bacteria.

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Supplementary Information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Force can overcome object geometry in the perception of shape through active touch

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Haptic (touch) perception normally entails an active exploration of object surfaces over time. This is called active touch^{1–3}. When exploring the shape of an object, we experience both geometrical⁴ and force cues. For example, when sliding a finger across a surface with a rigid bump on it, the finger moves over the bump while being opposed by a force whose direction and magnitude are related to the slope of the bump⁵. The steeper the bump, the stronger the resistance. Geometrical and force cues are correlated, but it has been commonly assumed that shape perception relies on object geometry alone. Here we show that regardless of surface geometry, subjects identified and located shape features on the basis of force cues or their correlates. Using paradoxical stimuli, for example combining the force cues of a bump with the geometry of a hole, we found that subjects perceived a bump. Conversely, when combining the force cues of a hole with the geometry of a bump, subjects typically perceived a hole.

In two experiments, human subjects explored surfaces by touch