

## Nickel-resistant bacteria isolated in human microbiome

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### Abstract

Nickel-resistant bacteria have been isolated so far only in contaminated soils and wastewaters polluted with different industrial sources. The aim of our study was to determine if nickel-resistant bacteria could also be isolated from human samples. In this brief communication, we describe how we were able to isolate human bacterial strains that grew without oxygen and in the presence of high concentrations of nickel. The identification was made by phenotypic and genetic techniques. The bacterial sequences have been deposited in the NCBI database repository. Our finding shows that there are several different heavy-metal-tolerant bacteria in humans that should be considered for further studies.

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### Background

Nickel is the 24th most abundant element in the earth and it is found in all environmental media: air, soil, sediment and water [1]. Nickel plays a fundamental role for a variety of metabolic

processes [2]. From an ecological perspective, nickel is an essential micronutrient for all higher plants and is important for seed germination [3–5]. However, although nickel in low nanomolar amounts (3–4 nM) is fundamental to many biological processes, at higher concentrations it causes damage. Soils may become contaminated by the accumulation of heavy metals through the emissions from rapidly expanding industrial areas, sewage sludge, wastewater irrigation and atmospheric deposition.

In human health, nickel in watches and jewellery has long been associated with eczema and contact dermatitis, and nickel contained in foods has been recently reported as responsible for weight gain, metabolic abnormalities and carcinogenicity [6–12].

An excess of nickel causes a perturbation of protein function and is toxic for eukaryotic cells and bacteria. Bacteria, evolved in metal-contaminated soils, appear to act as ‘chelating agents’ that not only ‘tolerate’ nickel in the classical sense (i.e. antibiotic-tolerant bacteria), but also thrive on the excess nickel, which results in its degradation and removal from the environment [13–16].

Although nickel-resistant bacteria in the environment have been extensively studied, essentially in humans no specific study has been undertaken to prove the presence of analogous nickel-resistant bacteria, despite the fact that we are constantly exposed to nickel.

The aim of this study was to investigate the potential existence of ‘nickel-tolerant’ bacteria in the human microbiome.

### Materials and methods

#### Sampling

Approval for this study was obtained from the IRB of the Italian Red Cross (No. 0053214).

Fresh stool samples were collected from ten volunteers, five of whom had an increased body mass index ( $>26 \text{ kg/m}^2$ ) and a documented nickel allergy. Each subject provided a fresh stool sample in a stool container on site. Within 30 min after defecation, the faecal samples were immediately stored at 4°C and processed within 2 h.

#### Isolation and screening of nickel-resistant bacteria

Stool cultures were made at different nickel sulphate concentrations in both aerobic and anaerobic conditions. A stock solution of nickel sulphate 250 mM was made by dissolving 66 g of  $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$  in 1 L of water in a laminar flow hood. A Fecal-Swab was turned into the stool and stool cultures were made in

**TABLE 1.** Biochemical characteristics of a clinical nickel-resistant bacterium (KY242648) isolated in 100% of obese subjects

<u>APPA</u>	:	<u>ADO</u>	:	<u>PyrA</u>	:	<u>IARL</u>	:	<u>dCEL</u>	:	<u>BGAL</u>	±
H2S	-	BNAG	-	AGLTp	-	dGLU	±	GGT	-	OFF	±
BGLU	-	dMal	±	dMAN	±	dMNE	±	BXYL	-	BAlap	-
ProA	-	LIP	-	PLE	-	TyrA	±	URE	-	dSOR	±
SAC	±	dTAG	-	dTRE	±	CIT	-	MNT	-	5KG	±
ILATk	-	AGLU	-	SUCT	±	NAGA	-	AGAL	±	PHOS	-
GlyA	-	ODC	±	LDC	±	IHISa	-	CMT	±	BGUR	±
O129R	±	GGAA	-	IMLTa	-	ELLM	-	ILATa	-		

liquid nutrient broth medium (Liofilchem, Roseto degli Abruzzi, Italy) supplemented with increasing concentrations of nickel sulphate ( $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ ) at 0.1, 0.2, 0.5, 1, 5, 10, 32 and 50 mM.

Stool cultures not supplemented with nickel were used as controls in each round of experiments. Liquid cultures were incubated at 37°C in both aerobic and anaerobic conditions. Bacterial growth was checked every day, over 10 days. Liquid bacterial cultures grown at the highest tolerable nickel concentration were seeded onto Luria–Bertani agar plates. The shape and colour of the colonies were examined under the microscope after Gram staining.

#### Identification of the bacterial isolates and 16 SrDNA gene amplification and sequencing

Microbiological identification and the measure of the various metabolic activities of the bacterial isolates was performed with Vitek 2 Compact instrumentation (bioMérieux, Marcy l'Étoile, France).

A bacterial suspension was adjusted to a McFarland standard of 0.5 in 2.5 mL of a 0.45% sodium chloride solution with an ATB 1550 densitometer. The time between preparation of the suspension and card filling was <30 min.

In addition to the biochemical characterization, a molecular identification of the nickel-tolerant bacteria was performed at the BMR genomics unit of Padua University. Bacterial genomic DNA was isolated by lysing a colony in 50 µl of PCR Buffer at 100°C for 5 min. A PCR of the first 530 bp of 16S ribosomal RNA (16S rDNA) of VI–V3 bacterial genes was performed with Primer 8FLP: AGTTTGATCCTGGCTCAG and Primer 534Rev: ATTACCGCGGCTGCTGG, using a Platinum PCR Super Mix High Fidelity Taq (Life Technologies, Carlsbad, CA, USA).

Conditions for the above primers for PCR were: –94°C for 2 min (one cycle), –94°C for 30 s, 55°C for 30 s, 68°C for 1 min (35 cycles), –68°C for 5 min (one cycle).

After gel analysis of the PCR products, cycle sequencing reactions were performed with Brilliant Dye terminator using the protocol from Nimagen (Nijmegen, The Netherlands). Extension products were purified and loaded into 3730xl with 50-cm array and POP-7™ Polymer for 3730/3730xl DNA

Analyzers. The consensus sequence, obtained by assembling the sequences file from Sanger sequencing, was compared with a nucleotide collection database using NCBI BLASTN program 2.6.1+ [17,18].

## Results

During the first 48 h of incubation, no bacterial growth was observed in the stool cultures supplemented with nickel.

Conversely, control stool cultures not supplemented with nickel, manifested the typical commensal flora growth after a few hours of incubation at 37°C, in both anaerobiosis and aerobiosis.

After 72 h of incubation, bacterial growth began in some of the human samples supplemented with nickel, but only in anaerobiosis and at a nickel concentration of 0.5 mM. Surprisingly, we realized that the growth occurred only in stools from participants with an increased body mass index and a nickel allergy.

After 96 h, bacterial growth could also be detected at 1 mM of nickel concentration, no viable organisms could be detected above this range of nickel concentration.

The growth of nickel-resistant bacteria was constantly characterized by a strict anaerobiosis and a prolonged lag phase, the duration of which depended on the nickel concentration in the media.

A typical growth curve for human nickel-resistant bacteria is described in the following link [https://drive.google.com/file/d/0B\\_YqsQvHtBOGOVZBSkhIMHJuRlk/view?usp=sharing](https://drive.google.com/file/d/0B_YqsQvHtBOGOVZBSkhIMHJuRlk/view?usp=sharing).

We isolated a strict anaerobic nickel-resistant bacterium in 100% of overweight subjects with a nickel allergy. The bacterium was Gram-negative and the genetic analyses of its 16S rDNA consensus sequence, revealed on its top hit a 99% similarity to multidrug-resistant *Shigella* variant. The biochemical behaviour of this isolate is reported in Table 1.

Concomitantly to the constant presence of this strictly anaerobic nickel-resistant isolate (NCBI KY242648), other anaerobic nickel-resistant bacteria were identified. *Enterococcus faecalis* and *Enterobacter* sp. were isolated in 50% and 16% from

**TABLE 2.** Top BLASTn hits for the nickel-resistant bacteria isolated from obese subjects

GenBank number for Human nickel-resistant bacteria	Top hit blastn 16S rDNA gene	Description	Score	Identities
Isolate KY242648	>gb CP012140.1	<i>Shigella flexneri</i> 4c strain 1205	893 bits (990)	497/498 (99%)
Isolate KY242649	>gb CP014949.1	<i>Enterococcus faecalis</i> strain LD33	928 bits (1028)	514/514 (100%)
Isolate KY242650	>gb CP014748.1	<i>Enterobacter aerogenes</i> strain FDAARGOS_139	893 bits (990)	495/495 (100%)

overweight allergic subjects, respectively. No nickel-resistant bacteria were identified in any of the lean subjects. The top hits of BLASTn search for the nickel-resistant bacteria are reported in Table 2.

## Results

We isolated nickel-resistant bacteria in obese humans, but not in any of the lean participants. Although the genetic 16 rRNA criteria indicated the genus of the bacterial isolates, their discriminant phenotypic and biochemical properties determined the novelty of the bacterial isolates as a new atypical strain. To separate species, phenotypic characters must also be identified and at least two phenotypic differences from existing species should be identified, if possible [19], (see <https://www.ncbi.nlm.nih.gov/books/NBK8406/>).

The human nickel-resistant bacteria were identified as *Shigella*, Enterococci and Enterobacter, but (i) they were strictly anaerobic, (ii) they only grew in the human samples from obese people, and (iii) they tolerated a maximum concentration of 1 mM nickel.

### Data availability

The 16 rRNA gene sequences of the identified human nickel-resistant bacteria have been deposited in GenBank NCBI with accession numbers KY24264848, KY24264849 and KY242650.

## Discussion and conclusion

Heavy-metal-resistant microorganisms occur naturally in primary contaminated soils. Their function and biological utility have been explored in agriculture and mining science. Nickel-resistant bacteria have also been viewed as useful scavengers evolved to eliminate the excess of heavy metals from the environment [20].

However, although interrelations have been described between nickel and human health, an effort to isolate nickel-resistant bacteria in humans has not previously been made. With a very simple and reproducible technique we were able to

isolate human nickel-resistant bacteria in the human microbiome, duplicating in humans similar findings of nickel-resistant bacteria isolated in contaminated soils and wastewaters polluted with effluents of metallurgic electroplating industrial sources [21,22].

The nickel-resistant strains isolated from human samples were mainly *Shigella*, Enterobacters and Enterococci: all potential pathogens.

*Shigella* are highly pathogenic bacteria that cause 1.1 million deaths and over 164 million communicable cases every year. Anaerobiosis primes these pathogens for invasion [23–25].

The Enterobacters were first linked to obesity after being found in high numbers in the guts of obese volunteers. These bacteria induced obesity in germ-free mice, which showed increased serum endotoxin level and aggravated inflammatory conditions [26].

Enterococci are usually gut commensals, but over the last few decades they have emerged as the leading causes of drug-resistant infection and important nosocomial multidrug-resistant organisms that affect debilitated patients undergoing prolonged hospitalization [27,28].

Gut microbes have been linked to chronic inflammation and altered fat storage in obese people [29–32]. In addition, the transfer of gut microbes from obese mice can transmit the obesity phenotype to lean mice [33]. In our analyses, nickel-resistant bacteria could be isolated only from the obese people, but not in any of the lean participants.

This finding supports recent studies where an excess of dietary nickel has been linked to overweight [34].

The lateral read from this exploratory study is that nickel does not just affect surface skin, but visceral skin also. An excess of heavy metals in the diet might promote a primary perturbation of the gut microbiota in ways that promote excess body weight and select nickel-resistant bacteria, which in turn might trigger inflammation in the intestinal tracts of the obese.

The transplant of nickel-resistant bacteria in germ-free mice could help in clarifying the possible role of these microbes in shaping the obese phenotype.

It can be concluded that there are several different heavy-metal-resistant bacteria in humans that should be considered for further studies.

## Conflicts of interest

No conflict of interest to declare.

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